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(54) Title: THERAPEUTIC DELIVERY OF CARBON MONOXIDE

(57) Abstract: Metal carbonyls are used to deliver CO having biological activity, for example vasodilatation and inhibition of transplant rejection. The metal of the carbonyl is typically of groups 7 to 10, e.g. Fe and Ru. The carbonyl preferably has one or more ligands other than CO, such as amino acids, to modulate the CO release property and solubility.

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# Therapeutic Delivery of Carbon Monoxide

FIELD OF THE INVENTION

The present invention relates to pharmaceutical

5    compositions and compounds for the therapeutic delivery

of carbon monoxide to humans and other mammals.  Another

use of the compositions and compounds is in organ

perfusion.

10   BACKGROUND OF THE INVENTION

Carbon monoxide (CO) is, by common definition, a

colorless, odorless, tasteless, non-corrosive gas of

about the same density as that of air and is the most

commonly encountered and pervasive poison in our

15   environment. It is generally produced by the incomplete

combustion of fossil fuels such as natural gas, propane,

coal, gasoline and wood. In the atmosphere, the average

global levels are estimated to be 0.19 parts per million

(p.p.m.), 90% of which comes from natural sources

20   including ocean micro-organism production, and 10% of

which is generated by human activity. Thus, inhalation

of even small quantities of CO is inevitable for living

organisms.

Depending on the extent and time of exposure, CO is

25   capable of producing a myriad of debilitating and

harmful residual effects to the organism (1). The most

immediate of these effects, and perhaps the most

notorious one, is binding to hemoglobin in the blood

stream, which rapidly decreases the oxygen transport

30   capability of the cardiovascular system. Paradoxically,

more than half a century ago it was found that CO is

constantly formed in humans in small quantities (2), and

that under certain pathophysiological conditions this

endogenous production of CO may be considerably
increased (3-5). The discovery that hemoglobin, a heme-
dependent protein, is required as substrate for the
production of CO in vivo (6,7) and the identification of

5     the enzyme heme oxygenase as the crucial pathway for the
generation of this gaseous molecule in mammals (8) set
the basis for the early investigation of an unexpected
and still unrecognized role of CO in the vasculature
(9). The succeeding cloning (10) and characterization of

10    constitutive (HO-2) and inducible (HO-1) isoforms of
heme oxygenase (11-13) as well as studies on the
kinetics and tissue distribution of these enzymes (14)
started to reveal a major importance of this pathway in
the physiological degradation of heme. That is, the end

15    products of heme degradation (CO, biliverdin and
bilirubin) might possess, after all, crucial biological
activities (15-17).

      With regard to the cardiovascular system, the
recognition that CO possesses vasodilatory properties

20    (18-20) is, perhaps, the most significant evidence in
favor of a pharmacological function of CO. Although the
molecular mechanisms and the chemical modifications that
are required to transduce the signals mediated by CO
into a specific biological effect need to be fully

25    elucidated, convincing scientific reports have recently
highlighted the signaling properties of endogenously
generated CO (21-24).

      Experimental studies on the physiological effects
of nitric oxide (NO) have been facilitated by the

30    development of a wide variety of organic compounds that
spontaneously release NO and can be easily acquired to
reproduce a physiological or pathophysiological function
of NO. There is now abundant literature on the different

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types of NO donors and NO-releasing agents that,
depending on their stability and half-life, can be used
in disparate *in vitro* and *in vivo* models to simulate the
biological activity of this important signaling molecule

5       (25,26). In clinical practice, compounds that deliver NO
into the circulation such as sodium nitroprusside and
glyceryl trinitrate are used to lower blood pressure and
treat certain cardiovascular diseases (27). Drugs
containing a functional NO group that can selectively

10      target an organ or tissue are currently being developed
or are under clinical trials for the treatment of
specific pathophysiological states (28,29). However, to
date no compounds capable of delivering CO
therapeutically have been identified.

15          US Patent 5,882,674 proposes administration of CO
via transdermal delivery systems containing metal
carbonyl complexes such as iron pentacarbonyl and iron
enneacarbonyl. However, since this document provides no
experimental data, and no description of specific

20      devices, it is not clear how this proposal can be made
to work. In particular it is not stated whether the
iron carbonyl complex is intended to be absorbed from
the patch, to release CO within the body, or whether the
complex breaks down within the patch to release CO which

25      then enters the bloodstream after absorption through the
skin. If, and to the extent that, this document is
considered to make available pharmaceutical devices,
compositions and methods for the practical and effective
delivery of carbon monoxide *in vivo*, such devices,

30      compositions and methods are excluded from the scope of
the present invention.

         Amongst literature relating to metal carbonyls,
WO98/48848 describes facial metal tricarbonyl compounds

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and their use in the labelling of biologically active
substrates.  The metals, preferably radionuclides, are
of Group 7, the metals identified being Mn, $^{99m}$Tc, $^{186}$Re
and $^{188}$Re.  The compounds fac-[M(CO)$_3$(OH$_2$)$_3$]$^+$ where M is

5    the metal are proposed for labelling of biologically
active substrates, as a result of which metal carbonyl
compounds having a variety of biologically active
ligands are obtained.  In the examples radioactive Tc is
used.  The document describes preparation of diagnostic

10   and therapeutic compositions but no therapeutic
composition is specifically disclosed, nor is any
treatment of any condition by therapy mentioned.  There
is no disclosure of use of the compounds for delivering
carbon monoxide to physiological targets.  If, and to

15   the extent that, this document is regarded as disclosing
a therapeutic use or mode of therapeutic administration
of the carbonyl compounds, that subject matter is
excluded from the scope of the present invention.
Preferably the present invention excludes use of the

20   facial carbonyl compounds disclosed in this document in
any event.
        WO 91/01128 and WO 91/01301 describe compositions
for treating skin to repair the effects of photoaging by
topical application or to treat acne or psoriasis by

25   topical or oral administration.  The active compounds
are polyene esters and iron carbonyl complexes thereof.
Specifically the iron of iron tri-carbonyl is
coordinated to the polyene chain.  No reason for
including the iron carbonyl is mentioned.  Insofar as

30   therapeutic uses or compositions of carbonyl compounds
are disclosed in these two documents, such uses and
compositions are specifically excluded from the scope of
the present invention.

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WO 98/29115 describes compositions and methods for relaxing smooth muscle in a warm-blooded animal by administering certain transition metal nitrosyl compounds.  Treatments of hypertension, angina pectoris, congestive heart failure and impotence are mentioned. Some of the compounds contain, in addition to NO, CO as a ligand.  Specifically the CO-containing compound has the formula $L_3M(NO)_yX_{3-y}$ where L is a two-electron Lewis base or $L_3$ is a six-electron Lewis base, M is a Group 6 or 8 transition metal and when y is 1, X is carbon monoxide.  The essential teaching of this document is concerned with the therapeutic effect of nitrosyl complexes.  There is no disclosure that the CO ligand, when present, has any therapeutic effect by delivery of CO to a physiological target.  The CO-containing metal nitrosyl complexes disclosed in it are excluded from the novel metal carbonyls of the present invention and their uses for treatments mentioned are also excluded from the present invention.  Preferably transition metal nitrosyl complexes containing CO are excluded from the scope of the present invention in any event.

HU-B-211084 describes a composition, which is for oral administration, for the fortification of bones containing calcium phosphate, at least one calcium salt of an organic acid and optionally iron pentacarbonyl. The present invention does not extend to the use of iron pentacarbonyl in combination with calcium compounds as specified in this document in connection with the therapeutic uses and modes of administration described there, and preferably does not extend to the use of iron carbonyls and complexes including iron and CO in combination with calcium phosphates and/or calcium salts of organic acids in any event.

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WO 95/05814 (US 6284752) and WO 00/56743 both disclose a very wide range of metal complexes, for use in treatment of disease relating to the overproduction of reactive oxygen species, particularly overproduction

5   of NO.  The stated aim is to modulate NO levels in the body by scavenging, or removing, NO *in situ*.  The ex-vivo test data are stated to show that vasconstriction is a direct result of the removal of endogenous nitric oxide.  Carbon monoxide is mentioned as a possible

10  ligand, but no example of a complex containing carbon monoxide is given and no effect is attributed to CO. Insofar as these documents are considered to disclose practical use of a complex containing CO for the specified purpose, such use does not form part of the

15  present invention.


SUMMARY OF THE INVENTION

As exemplified by the experimental data detailed below, the present inventors have found that metal

20  carbonyl compounds can be used to deliver CO to a physiological target so as to provide physiological effect.

Accordingly the present invention provides a pharmaceutical composition, for delivery of carbon

25  monoxide to a physiological target, comprising a metal carbonyl compound or pharmaceutically acceptable salt thereof and at least one pharmaceutically acceptable carrier, wherein the metal carbonyl makes available CO suitable for physiological effect by at least one of the

30  following means:

1)  CO derived by dissociation of the metal carbonyl is present in the composition in dissolved form;

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2) on contact with a solvent the metal carbonyl releases CO;

3) on contact with a tissue, organ or cell the metal carbonyl releases CO;

4) on irradiation the metal carbonyl releases CO.

Certain metal carbonyl compounds are capable of releasing CO on contact with a suitable solvent. When the pharmaceutical composition is to be administered in liquid form, this solvent may form a component part of the pharmaceutical composition. Thus in this aspect of the invention, the pharmaceutical composition contains CO derived from the metal carbonyl in dissolved form. The conditions under which the carbonyl compound is dissolved in the solvent during preparation of the pharmaceutical may be controlled such that the CO thus released is retained in solution. This may be facilitated where an equilibrium exists between the dissociated components and the undissociated carbonyl.

The dissociated components of the parent carbonyl may themselves be metal carbonyl complexes capable of releasing further CO. For example, when $[Ru(CO)_3Cl_2]_2$ is dissolved in DMSO, CO is liberated into solution, and a mixture of tri-carbonyl and di-carbonyl complexes is formed, and these themselves may be capable of releasing further CO.

In a further aspect of the invention, the pharmaceutical composition may not itself contain dissolved CO, but may be prepared such as to release CO on contact with a suitable solvent or medium. For example, the composition may contain a metal carbonyl compound capable of releasing CO on contact with water, e.g. on contact with an aqueous physiological fluid, such as blood or lymph. Alternatively, the

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pharmaceutical composition may be intended to be
dissolved in water prior to administration.  Such
pharmaceutical compositions may be prepared in solution
or in solid form, such as in tablet form.  If they are

5    in solution form, they will typically be prepared in a
solvent which does not support dissociation of the metal
carbonyl compound, such that release of CO takes place
only on contact with the appropriate solvent.

Alternatively or additionally, release of CO from

10   the complex can be stimulated by reaction with a ligand
in solution which for example replaces one of the
ligands of the complex leading to loss of CO from the
complex.

In another aspect of the invention the

15   pharmaceutical composition may contain a metal carbonyl
compound which releases CO on contact with a tissue,
organ or cell.  It is shown below that certain metal
carbonyl compounds do not release CO to solution but are
nevertheless capable of releasing CO to physiological

20   cellular materials or tissues, such as vascular
endothelium.  For example, $[Fe(SPh)_2(2,2'-$
bipyridine$)(CO)_2]$ is shown below not to release CO to
myoglobin in solution, but is nevertheless capable of
promoting dilatation of pre-contracted aortic rings.

25   Without wishing to be limited by any particular theory,
it is thought that CO may be released from such
compounds as a result of an oxidation-reduction
reaction, mediated by cellular components such as
cytochromes.

30   However the invention is not limited to a redox
reaction as a mechanism for CO release, since loss of at
least a first CO from the complex may occur without
redox.

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In a further aspect of the invention, the
pharmaceutical composition may contain a metal carbonyl
compound which releases CO on irradiation. The compound
may be irradiated prior to administration, for example
5     to produce a solution of dissolved CO, or may be
irradiated *in situ* after administration. It is
contemplated that such compositions may be used to
provide controlled, localised release of CO. For
example a pharmaceutical composition of this type may be
10    administered during surgery, and CO released
specifically at a site in need thereof, e.g. to induce
vasodilation, by localised irradiation by means of a
laser or other radiant energy source, such as UV rays.

Typically the pharmaceutical compositions of the
15    present invention release CO such as to make it
available to a therapeutic target in dissolved form.
However, in some circumstances CO may be released from a
metal carbonyl directly to a non-solvent acceptor
molecule.

20         It will be apparent that pharmaceutical
compositions according to the present invention may be
capable of delivering CO therapeutically through one or
more of the above described modes of action.

Typically the metal carbonyl compound comprises a
25    complex of a transition metal, preferably a transition
metal from group 7 or groups 8 to 10 (in this
specification the groups of the periodic table are
numbered according to the IUPAC system from 1 to 18).
The number of carbonyl ligands is not limited, provided
30    at least one carbonyl ligand is present. The preferred
metals are transition metals of lower molecular weight,
in particular Fe, Ru, Mn, Co, Ni, Mo and Rh. Two other
metals which may be used are Pd and Pt. In the metal

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carbonyl complexes used in the invention, the metal is
typically in a low oxidation state, i.e. O, I or II.
For the metals preferred, the oxidation states are
typically not higher than $Fe^{II}$, $Ru^{II}$, $Mn^{I}$, $Co^{II}$ preferably

5    $Co^{I}$, $Rh^{III}$ preferably $Rh^{I}$, $Ni^{II}$, $Mo^{II}$.  The metal is
preferably not a radionuclide.  Fe is one particularly
suitable metal, since Fe is present in quantity in
mammals.

The metal carbonyl compounds may be regarded as

10   complexes, because they comprise CO groups coordinated
to a metal centre.  However the metal may be bonded to
other groups by other than coordination bonds, e.g. by
ionic or covalent bonds.  Thus groups other than CO
which form part of the metal carbonyl compound need not

15   strictly be "ligands" in the sense of being coordinated
to a metal centre via a lone electron pair, but will be
referred to herein as "ligands" for ease of reference.

Thus, the ligands to the metal may all be carbonyl
ligands, as e.g. in $[Mn_2(CO)_{10}]$.  Alternatively, the

20   carbonyl compound may comprise at least one modulatory
ligand.  By this is meant a ligand which is not CO, but
which modulates a particular property of the complex,
such as the tendency to release CO, solubility,
hydrophobicity, stability, electrochemical potential,

25   etc.  Thus suitable choices of ligand may be made in
order to modulate the behaviour of the compound.  For
example it may be desirable to modulate the solubility
of the compound in organic and/or aqueous solvents, its
ability to cross cell membranes, its rate of release of

30   CO on contact with a particular solvent or cell type, or
on irradiation, etc.

Such ligands are typically neutral or anionic
ligands, such as halide, or derived from Lewis bases and

having N, P, O or S or a conjugated carbon group as the
coordinating atom(s). Preferred coordinating atoms are
N, O and S. Examples include, but are not limited to,
sulfoxides such as dimethylsulfoxide, natural and
5   synthetic amino acids and their salts for example,
glycine, cysteine, and proline, amines such as $NEt_3$ and
$H_2NCH_2CH_2NH_2$, aromatic bases and their analogues, for
example, bi-2,2'-pyridyl, indole, pyrimidine and
cytidine, pyrroles such as biliverdin and bilirubin,
10   drug molecules such as YC-1 (2-(5'-hydroxymethyl-2'-
furyl)-1-benzylindazole), thiols and thiolates such as
EtSH and PhSH, chloride, bromide and iodide,
carboxylates such as formate, acetate, and oxalate,
ethers such as $Et_2O$ and tetrahydrofuran, alcohols such as
15   EtOH, and nitriles such as MeCN. Particularly preferred
are coordinating ligands, such as amino acids, which
render the carbonyl complex stable in aqueous solution.
Other possible ligands are conjugated carbon groups,
such as dienes. One class of ligands which can provide
20   metal carbonyl compounds of use in this invention is
cyclopentadiene ($C_5H_5$) and substituted cyclopentadiene.
The substituent group in substituted cyclopentadiene may
be for example an alkanol, an ether or an ester, e.g. –
$(CH_2)_nOH$ where n is 1 to 4, particularly $-CH_2OH$, $-(CH_2)_nOR$
25   where n is 1 to 4 and R is hydrocarbon preferably alkyl
of 1 to 4 carbon atoms and $-(CH_2)_nOOCR$ where n is 1 to 4
and R is hydrocarbon preferably alkyl of 1 to 4 carbon
atoms. The preferred metal in such a cyclopentadiene or
substituted cyclopentadiene carbonyl complex is Fe.
30   Preferably the cyclopentadiene carbonyl complex is
cationic, being associated with an anion such as
chloride.

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As mentioned above certain metal nitrosyl complexes disclosed in WO 98/29115 and their uses disclosed are excluded from the present invention, and preferably the invention does not extend to metal carbonyl complexes containing NO (nitrosyl) in any event.  Furthermore as mentioned above certain iron carbonyl complexes disclosed in WO 91/01128 and WO 91/01301 and their uses disclosed therein are excluded from the present invention.  Preferably the invention does not extend to topical or oral administration of iron carbonyl polyene complexes, nor to these complexes in themselves.

A further exclusion from the present invention are the Mn and radionuclide complexes disclosed in WO 98/48848.  Preferably the present invention excludes therapeutic use of these Mn complexes.  Preferably the invention excludes carbonyls of radioactive metals, in any case.

CO is suggested to act at least in part through the stimulation of guanylate cyclase activity.  Thus the metal carbonyl compound may desirably comprise ligands which modulate the effect of CO on guanylate cyclase. For example, the drug YC-1 (3-(5'-hydroxymethyl-2'-furyl)-1-benzylindole) is thought to enhance stimulation of guanylate cyclase by CO.  Thus incorporation of ligands such as YC-1 or derivatives thereof into the metal carbonyl compounds can alter or enhance the biological effects of the released CO.

Thus the properties of pharmaceutical compositions of the present invention may be tailored as required by appropriate choice of metal centres and number and type of associated ligands in the metal carbonyl compound.

The metal carbonyl compound may further comprise a targeting moiety, to facilitate release of CO at an

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appropriate site.  The targeting moiety is typically
capable of binding a receptor on a particular target
cell surface, in order to promote release of CO at the
required site.  The targeting moiety may be a part of a
5      modulating ligand capable of binding to a receptor found
on the surface of the target cells, or may be derived
from another molecule, such as an antibody directed
against a particular receptor, joined to the complex by
a suitable linker.

10        The present invention also provides a
pharmaceutical composition for delivery of CO,
comprising as active ingredient a compound of the
formula $M(CO)_x A_y$ where x is at least one, y is at least
one, M is a metal, A is an atom or group bonded to M by
15     an ionic, covalent or coordination bond, and, in the
case where y>1, each A may be the same or different, or
a pharmaceutically acceptable salt of such a compound.
Typically, M is a transition metal, particularly of
group 7 or groups 8 to 10, and A may be selected from
20     halogens, groups having N, P, O or S atoms providing
lone electron pairs for coordination bonding to M, and
conjugated carbon groups.  More details of preferred
metals and ligands are given above.  The carbonyl
complex should be pharmaceutically acceptable, in
25     particular non-toxic or of acceptable toxicity at the
dosage levels envisaged.

The pharmaceutical compositions of the present
invention typically comprise a pharmaceutically
acceptable excipient, carrier, buffer, stabiliser or
30     other materials well known to those skilled in the art.
Such materials should be non-toxic and should not
interfere unduly with the efficacy of the active
ingredient.  The precise nature of the carrier or other

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material may depend on the route of administration, e.g. oral, intravenous, subcutaneous, nasal, intramuscular, intraperitoneal, or suppository routes.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant or a slow-release polymer. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included. Pharmaceutically acceptable amounts of other solvents may also be included, in particular where they are required for dissolving the particular metal carbonyl compound contained in the composition.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will typically be in the form of a parenterally acceptable solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required. Delivery systems for needle-free injection are also known, and compositions for use with such systems may be prepared accordingly.

Administration is preferably in a prophylactically effective amount or a therapeutically effective amount

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(as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual.  The actual amount administered, and rate and time-course of

5     administration, will depend on the nature and severity of what is being treated.  Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the

10    disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences,

15    16th edition, Osol, A. (ed), 1980.

When formulating pharmaceutical compositions according to the present invention, the toxicity of the active ingredient and/or the solvent must be considered. The balance between medical benefit and toxicity should

20    be taken into account.  The dosages and formulations of the compositions will typically be determined so that the medical benefit provided outweighs any risks due to the toxicity of the constituents.

There is further provided a method of introducing

25    CO to a mammal comprising the step of administering a pharmaceutical composition according to the present invention.  CO is thought to act at least in part through stimulation or activation of guanylate cyclase. CO is thought to have functions as, inter alia, a

30    neurotransmitter and a vasodilating agent.  Accordingly there is provided a method of delivering CO to a mammal for stimulation of guanylate cyclase activity.  There is further provided a method of delivering CO to a mammal

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for stimulating neurotransmission or vasodilation.
However the present applicants do not wish to be bound
by theory and do not exclude the possibility that CO
operates by other mechanisms.

5      The heme oxygenase 1 (HO-1) pathway is thought to
represent a pivotal endogenous inducible defensive
system against stressful stimuli including UVA
radiations, carcinogens, ischaemia-reperfusion damage,
endotoxic shock and several other conditions
10    characterised by production of oxygen free radicals (30-
32). The protective effect of HO-1 is attributed to the
generation of the powerful antioxidants biliverdin and
bilirubin and the vasoactive gas CO.  Expression of HO-1
has been linked with cardiac xenograft survival (33),
15    suppression of transplant arteriosclerosis (34) and
amelioration of post-ischemic myocardial dysfunction
(35). HO-1 has also been directly implicated in the
resolution phase of acute inflammation in rats (36).
Other pathological situations, such as haemorrhagic
20    shock in brain and liver as well as sepsis (37-39), are
characterized by induction of the HO-1 gene, which seems
to play a crucial role in counteracting the vascular
dysfunction caused by these pathophysiological states.
Increased generation of CO as a consequence of HO-1
25    induction markedly affects vessel contractility and
diminishes acute hypertension in the whole organism
(23,40). Exposure of animals to ambient air containing
low concentrations of CO or transfection of the HO-1
gene results in protection against hyperoxia-induced
30    lung injury *in vivo*, a mechanism mediated by attenuation
of both neutrophil inflammation and lung apoptosis (cell
death) (41,42). Exogenous CO gas also has the ability to
suppress pro-inflammatory cytokines and modulate the

expression of the anti-inflammatory molecule, IL-10, both *in vitro* and *in vivo* (43). Therefore administration of CO in accordance with the invention may be used for treatment of any of these conditions, for modulation of

5    inflammatory states and regression of other pathophysiological conditions including cancer.

Accordingly there is provided a method of introducing CO to a mammal comprising the step of administering a pharmaceutical composition according to

10   the present invention, for treatment of hypertension, such as acute, pulmonary and chronic hypertension, radiation damage, endotoxic shock, inflammation, inflammatory-related diseases such as asthma and rheumatoid arthritis, hyperoxia-induced injury,

15   apoptosis, cancer, transplant rejection, arteriosclerosis, post-ischemic organ damage, myocardial infarction, angina, haemorrhagic shock, sepsis, penile erectile dysfunction and adult respiratory distress syndrome.

20   The present invention also provides the use of a metal carbonyl compound as herein described in the manufacture of a medicament for delivering CO to a physiological target, particularly a mammal, to provide a physiological effect, e.g. for stimulating

25   neurotransmission or vasodilation, or for treatment of any of hypertension, such as acute, pulmonary and chronic hypertension, radiation damage, endotoxic shock, inflammation, inflammatory-related diseases such as asthma and rheumatoid arthritis, hyperoxia-induced

30   injury, apoptosis, cancer, transplant rejection, arteriosclerosis, post-ischemic organ damage, myocardial infarction, angina, haemorrhagic shock, sepsis, penile erectile dysfunction and adult respiratory distress

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syndrome. Such medicaments may be adapted for administration by an oral, intravenous, subcutaneous, nasal, inhalatory, intramuscular, intraperitoneal or suppository route. Preferably the present invention

5    excludes delivery of a metal carbonyl or a decomposition product thereof to an organism through the skin or mucosa.

The invention further provides use of the metal carbonyls here described in treatment, e.g. by

10   perfusion, of a viable mammalian organ extracorporeally, e.g. during storage and/or transport of an organ for transplant surgery. For this purpose, the metal carbonyl is in dissolved form, preferably in an aqueous solution. The viable organ may be any tissue containing

15   living cells, such as a heart, a kidney, a liver, a skin or muscle flap, etc.

The invention also consists in a metal carbonyl compound of the formula

$M(CO)_x A_y B_z$ where

20   M is Fe, Co or Ru,

x is at least one,

y is at least one,

z is zero or at least one,

each A is a ligand other than CO and is monodentate

25   or polydentate with respect to M and is selected from the amino acids

alanine

arginine

asparagine

30   aspartic acid

cysteine

glutamic acid

glutamine

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glycine

histidine

isoleucine

leucine

5              lysine

methionine

phenylalanine

proline

serine

10             threonine

tryptophan

tyrosine

valine

$O(CH_2COO)_2$ and

15      $NH(CH_2COO)_2$, and

B is optional and is a ligand other than CO,

excluding $Fe(CO)_x A_y$ where A is cysteine or an ester

of cysteine and $Ru(CO)_x A_y$ where A is proline.

x is preferably 3, y is preferably 1 and z is

20      preferably 1.

The term amino acid here used includes the species

obtained by loss of the acidic hydrogen, such as

glycinato.

$B_z$ represents one or more optional other ligands.

25      There are no particular limitations on B, and ligands

such as halides, e.g. chloride, bromide, iodide, and

carboxylates, e.g. acetate may be used.

M is selected from Fe, Ru and Co. These metals are

preferably in low oxidation states, as described above.

30      Use of the known iron compounds $[Fe(SPh)_2(2,2'-$

bipyridine$)(CO)_2]$ and $[Fe(SPh)_2(NH_2CH_2CH_2NH_2)(CO)_2]$ is also

envisaged in this invention.

20

It is further considered that, in place of the
metal carbonyl compounds discussed above, the
pharmaceutical compositions of the present invention may
comprise oxalate compounds, formic acid, or formate

5    compounds, which may likewise deliver CO to a
physiological target.  For example, bis-(2,4-
dinitrophenyl) oxalate is known to decompose in water to
liberate CO into solution.  Therefore the present
invention further provides a pharmaceutical composition,

10   for delivery of carbon monoxide to a physiological
target, comprising formic acid, a formate, a formate
ester or amide, an oxalate, or an oxalate ester or
amide, or a pharmaceutically acceptable salt thereof and
at least one pharmaceutically acceptable carrier,

15   wherein the formic acid, formate or oxalate or amide or
ester makes available CO suitable for physiological
effect.

It is thought that the nitrophenyl groups of bis-
(2,4-dinitrophenyl) oxalate are good leaving groups,

20   because of the electron-withdrawing effects of the nitro
groups, and that this may promote the decomposition of
the oxalate to yield CO.

It is therefore considered that oxalates or
formates having in which the acid groups are linked,

25   e.g. by an ester bond, to aromatic groups with electron-
withdrawing substituents, such as tosyl groups, are
particularly suitable for use in pharmaceutical
compositions according to the present invention.

There is further provided a method of introducing

30   carbon monoxide to a mammal comprising the step of
administering a pharmaceutical composition comprising
formic acid, a formate, a formate ester or amide or an
oxalate, an oxalate ester or amide, or a

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pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable carrier.

All the above discussion and disclosure relating to metal carbonyl compounds is also considered to relate to
5   formic acid, formates, oxalates and formate or oxalate amides and esters.

Throughout this application, references to medical treatment are intended to include both human and veterinary treatment, and references to pharmaceutical
10  compositions are accordingly intended to encompass compositions for use in human or veterinary treatment.


INTRODUCTION OF THE DRAWINGS

Experimental data illustrating the present
15  invention will now be described by reference to the accompanying figures, in which:

Figure 1 shows apparatus for measuring release of CO by metal carbonyl complexes on irradiation and structures of $[Mn_2(CO)_{10}]$ and $[Fe(CO)_5]$.

20      Figure 2 shows deoxy-myoglobin and CO-myoglobin absorption spectra.

Figure 3 shows NMR spectra illustrating the dissolution of $[Ru(CO)_3Cl_2]_2$ in DMSO.

Figure 4 shows viability data for cells treated
25  with metal carbonyl compounds.

Figure 5 shows relaxation of aortic rings on treatment with metal carbonyl complexes.

Figure 6 shows the effects of various treatments on perfused rat hearts.

30      Figure 7 shows expression of heme oxygenase 1 in rat hearts.

Figure 8 shows the effects of various treatments on rat mean arterial pressure.

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Figures 9a to 9f are tables presenting CO release data of metal carbonyl complexes.

Figure 10 is a graph showing survival rates in a transplant rejection study described below.

Figure 11 is a graph of nitrite produced in a study of NO production in macrophages described below.

Figure 12 is graphs of cell viability in the study of NO production in macrophages.

## EMBODIMENTS OF THE INVENTION AND EXPERIMENTAL DATA

For the experiments here described, iron pentacarbonyl, [Fe(CO)$_5$], dimanganese decacarbonyl, [Mn$_2$(CO)$_{10}$], tricarbonyldichlororuthenium (II) dimer, [Ru(CO)$_3$Cl$_2$]$_2$, and ruthenium(III) chloride hydrate, RuCl$_3$, were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). Other carbonyl complexes have been synthesized, as described below. Stock solutions of metal carbonyl complexes were prepared fresh prior to each experiment by dissolving the compounds in dimethyl sulfoxide (DMSO), water or saline. Hemin (ferriprotoporphyrin IX chloride) and tin protoporphyrin IX (SnPPIX) were from Porphyrin Products Inc. (Logan, Utah, USA). Stock solutions of both porphyrins were prepared by dissolving the compounds in 0.1 M NaOH and then adjusting the pH to 7.4 by addition of 0.01 M phosphate buffer. The guanylate cyclase inhibitor, [1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one] (ODQ), was obtained from Alexis Corporation (Bingham, Nottingham, UK) and polyclonal rabbit anti-HO-1 antibodies were purchased from Stressgen (Victoria, Canada). Horse heart myoglobin, N$^G$-nitro-L-arginine methyl ester (L-NAME) and all other reagents were from Sigma, unless otherwise specified.

23

All data are expressed as mean ± s.e.m. Differences
between the groups analysed were assessed by the
Student's two-tailed *t*-test, and an analysis of variance
(ANOVA) was performed where more than two treatments
5    were compared. Results were considered statistically
significant at $P<0.05$.


## A.    Detection of CO liberated from transition metal carbonyl complexes.

10        The release of CO from metal carbonyl complexes was
assessed spectrophotometrically by measuring the
conversion of deoxymyoglobin (deoxy-Mb) to carbonmonoxy
myoglobin (MbCO). MbCO has a distinctive absorption
spectrum between 500 and 600 nm, and changes at 540 nm
15    were used to quantify the amount of CO liberated.
Myoglobin solutions (66 μM final concentration) were
prepared freshly by dissolving the protein in 0.04 M
phosphate buffer (pH 6.8). Sodium dithionite (0.1 %) was
added to convert myoglobin to deoxy-Mb prior to each
20    reading.  All the spectra were measured using a Helios α
spectrophotometer.

Direct addition of iron pentacarbonyl, [Fe(CO)$_5$], or
dimanganese decacarbonyl, [Mn$_2$(CO)$_{10}$], to myoglobin
solutions did not result in any appreciable formation of
25    carbonmonoxy myoglobin (MbCO) over time (data not
shown). This is consistent with the notion that these
two transition metal carbonyl complexes do not release
CO unless stimulated by light (44,45).  Therefore
release of CO was induced by exposing these metal
30    carbonyl complexes to a cold light source and allowing
the gas to diffuse through a membrane before reacting
with myoglobin as shown in Figure 1.

24

Five hundred microliters of iron pentacarbonyl ([Fe(CO)$_5$], 99.9%) or 1 ml of dimanganese decacarbonyl ([Mn$_2$(CO)$_{10}$], 13 mM in DMSO) (see also chemical structure) were placed as carbonyl solution 2 in a plastic tube 1. A cell culture insert 3 (Costar) was sealed on top in order to create two separate chambers with a 0.6 cm air space between the solution 2 and an insert membrane 6 (Anapore™ 0.4 µm). 1.5 ml of deoxy-Mb solution (66 µM) was placed in the insert which was covered with Parafilm™ 5. The carbonyl solution was then exposed to cold light from a source 7 to stimulate CO release, allowing the gas to diffuse through the membrane 6 into the myoglobin solution 4. Aliquots of the myoglobin solution 4 were taken at different times and the conversion of deoxy-Mb to MbCO measured spectrophotometrically.

The spectral change on transition from deoxy-Mb to MbCO was measured by bubbling CO gas to a solution of deoxy-Mb (Figure 2a). Upon illumination, [Fe(CO)$_5$] and [Mn$_2$(CO)$_{10}$] produced a similar change in the absorbance spectrum of myoglobin, with a gradual increase in MbCO formation observed over time; in both cases the distinctive identified spectra were the ones typical of MbCO (Figures 2b and 2c). Under the experimental conditions used, a complete saturation of the myoglobin solution was achieved by [Mn$_2$(CO)$_{10}$] (13 µmol/ml) in approximately 40 min of continuous exposure to light (Figure 2d).

Various metal carbonyl complexes were tested for their ability to elicit MbCO formation when added directly to a deoxy-Mb solution. To different extents, [Ru(CO)$_3$Cl$_2$]$_2$, [Ru(CO)$_2$(DMSO)$_2$Cl$_2$], [Ru(CO)$_3$Cl$_2$(cytosine)] and [Ru(CO)$_3$(glycinate)Cl] all released CO when added

25

directly to the Mb solution.  No MbCO was detected in the case of  [Fe(SPh)$_2$(2,2'-bipyridine)(CO)$_2$] and [Fe(SPh)$_2$(H$_2$NCH$_2$CH$_2$NH$_2$)(CO)$_2$], but as shown below both these compounds provided a vasodilatory effect.

5      Data for the tricarbonyldichlororuthenium (II) dimer [Ru(CO)$_3$Cl$_2$]$_2$ are shown in Figure 2e.  The metal carbonyl complex was solubilized in DMSO (9.7 mM stock solution), aliquots of 2 to 32 µl were added directly to 1 ml of deoxy-Mb solutions (66 µM) and absorption
10     spectrum determined immediately after mixing the samples by inversion.  A linear regression analysis of the saturation curve of MbCO revealed that for each mole of [Ru(CO)$_3$Cl$_2$]$_2$ approximately 0.7 moles of CO are liberated (Figure 2f).

15     Further data on release of CO measured by the same test procedure is described in section H below.


## B.  NMR studies of [Ru(CO)$_3$Cl$_2$]$_2$

       Further studies were conducted on the chemistry of
20     transition metal carbonyls using NMR spectroscopy.  The $^{13}$C NMR spectrum showed that [Ru(CO)$_3$Cl$_2$]$_2$ freshly dissolved in DMSO does not exist as a dimer; in fact, two distinct sets of signals corresponding to the known tri-carbonyl (**1**) and di-carbonyl (**2**) monomers (see
25     formulae in Fig. 3) could be identified. The NMR analysis reveals that, during the solubilization process, DMSO acts as a coordinated ligand to ruthenium thereby promoting the formation of the monomers.

       Figure 3a shows a 100.62 MHz $^{13}$C{$^1$H} NMR spectrum
30     taken during the first 23 min of the reaction between freshly prepared [RuCl$_2$(CO)$_3$]$_2$ and d$_6$-DMSO. The solution very slowly produced fine bubbles of a gas, presumably CO, implied by the formation of compound **2**. When the

experiment was repeated by dissolving initially the
metal complex in DMSO and then diluting with $CDCl_3$, the
assignment of the signals coincided with the published
$^{13}C(CO)$ chemical shifts of fac-[$RuCl_2(CO)_3(DMSO)$] (1, $\delta$

5    183.0, 186.8), cis, cis, trans-[$RuCl_2(CO)_2(DMSO)_2$] (2, $\delta$
185.0) and cis, cis, cis-[$RuCl_2(CO)_2(DMSO)_2$] (3, $\delta$
186.0, 191.9) (46). Figure 3b shows a 100.62 MHz $^{13}C\{^1H\}$
NMR spectrum taken after [$RuCl_2(CO)_3$]$_2$ in $d_6$-DMSO was
warmed at 50 °C for 5 min and left to accumulate

10   overnight. In addition to the peaks that could be
assigned to compounds 1, 2 and 3, there are carbonyl
signals at $\delta$ 187.9 and 190.5 due to unidentified
species.

        The detection of di-carbonyl monomers demonstrates

15   that CO is liberated; the $^{13}C$ NMR spectrum also suggests
that the ratio between compounds 1 and 2 is 40:60.

        In sections C and D below, we refer for convenience
to [$Ru(CO)_3Cl_2$]$_2$, but as explained here, when dissolved
in DMSO other species are actually present.

20

## C.    Effect of [Ru(CO)$_3$Cl$_2$]$_2$ on cell viability

        As there are no precedent studies on the use of
metal carbonyl complexes in biological systems, it was
necessary to evaluate the potential cytotoxic effect of

25   these compounds. Therefore, the viability of cells in
culture was determined after short or prolonged exposure
to various concentrations of metal carbonyls.

        Rat vascular smooth muscle cells were obtained from
the Coriell Cell Repository (Camden, NJ, USA) and grown

30   in Dulbecco's Minimal Essential Medium (MEM)
supplemented with 20% foetal calf serum, 2 x MEM
vitamins, 2 x MEM non-essential and essential amino
acids, penicillin (100 units/ml) and streptomycin (0.1

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mg/ml). Confluent cells were treated with different
concentrations of metal carbonyl (introduced as DMSO
solution - see section B) for various times and cell
viability was assessed using a colorimetric assay kit

5   from Promega (Madison, WI, USA) as previously described
(47) after 3 or 24 h incubation, or after 3 h exposure
to the agents followed by 21 h incubation in complete
media. Results are expressed as the mean ± s.e.m. of 6
independent experiments and differences were considered

10   statistically significant at $P < 0.05$ (*).

Exposure of $[Fe(CO)_5]$ to light gradually resulted in
deposition of a green-brown precipitate, and so
viability studies on this metal carbonyl were not
pursued. Nevertheless, $[Fe(SPh)_2(2,2'-bipyridine)(CO)_2]$

15   proved to elicit a marked vasodilatory effect (see
below).

As shown in Figure 4b, treatment of vascular smooth
muscle cells for 3 h with $[Ru(CO)_3Cl_2]_2$ (0-420 µM final
concentration) did not promote any detectable

20   cytotoxicity. Similarly, cell viability was well
preserved after exposure to this metal carbonyl for 3 h
followed by an additional 21 h incubation in complete
medium. A pronounced cytotoxic effect (>50% loss in cell
viability) was only apparent after prolonged exposure

25   (24 h) to very high concentrations (> 400 µM) of
$[Ru(CO)_3Cl_2]_2$.

Treatment of cells with the same amounts of vehicle
(DMSO) or equivalent molar concentrations of ruthenium
$(RuCl_3)$ did not cause any appreciable decrease in cell

30   viability over time (Figure 4a and 4c, respectively)
indicating that neither the vehicle nor the metal are
responsible for the observed cytotoxic effect of
$[Ru(CO)_3Cl_2]_2$.

28

In the case of $[Mn_2(CO)_{10}]$ (0-100 μM), no major cytotoxicity on smooth muscle cells was detected after exposure for 24 h in complete medium (data not shown).

5    **D.  Vasodilatory effect of CO released from $[Ru(CO)_3Cl_2]_2$**

It has previously been demonstrated that increased endogenous CO as a result of HO-1 induction in rat aortas markedly attenuates vasoconstriction (23). To investigate whether CO released from metal carbonyl

10   complexes evokes specific biological activities, we first assessed the effect of these complexes on vessel contractility using the isolated aortic ring model.

Transverse ring sections of thoracic aorta were isolated from male Lewis rats and suspended under a 2 g

15   tension in an organ bath containing oxygenated Krebs-Henseleit buffer at 37 °C as previously described (23). The relaxation response to cumulative doses of metal carbonyl (dissolved in DMSO – see section B) was assessed in aortic rings pre-contracted with

20   phenylephrine (3 μM). Control rings were similarly treated by adding equal doses of DMSO (vehicle) to the organ bath. Results are shown in Table 1 and Figure 5.

As shown in Figure 5, consecutive additions of $[Ru(CO)_3Cl_2]_2$ (222μM final concentration) to aortic rings

25   pre-contracted with phenylephrine elicited a rapid and significant vasodilatation (P<0.05); the extent of relaxation was already pronounced after the first addition of the compound (45% more than control). Interestingly, after extensive washing, the

30   phenylephrine-induced contraction was completely restored in control but not in $[Ru(CO)_3Cl_2]_2$-treated vessels indicating that this compound produces long-lasting effects.

29

The vasodilatory response mediated by metal carbonyls was almost totally abolished when reduced Mb (150µM), which avidly binds CO, was added to the buffer. Collectively, these findings are consistent with the
5  fact that CO released from metal carbonyls possesses vasoactive properties.

As shown in Table 1, $[Ru(CO)_2(DMSO)_2Cl_2]$ also produced vasodilatation although the effect was less pronounced compared to $[Ru(CO)_3Cl_2]_2$. Interestingly,
10  while $[Ru(CO)_3Cl_2(cytosine)]$ did not demonstrate any effect during the time duration of this experiment, $[Ru(CO)_3(glycinato)Cl]$ elicited significant vasodilatation which is consistent with the high release of CO detected with the MbCO assay. Notably,
15  $[Fe(SPh)_2(2,2'-bipyridine)(CO)_2]$ which did not release any detectable CO to myoglobin, was still very effective in promoting vasorelaxation. On the other hand, the effect of $[Fe(SPh)_2(H_2NCH_2CH_2NH_2)(CO)_2]$ was less evident.

## Table 1

| Treatment | % Relaxation | | |
|---|---|---|---|
| | *1st addition* | *2nd addition* | *3rd addition* |
| Vehicle (DMSO) | $5.7 \pm 0.9$ | $11.4 \pm 1.1$ | $18.1 \pm 2.5$ |
| $[Ru(CO)_3Cl_2]_2$ | $49.9 \pm 2.7*$ | $66.2 \pm 3.2*$ | $74.1 \pm 4.1*$ |
| $[Ru(CO)_3Cl_2]_2$ + Mb | $4.0 \pm 0.9^{\dagger}$ | $8.6 \pm 0.4^{\dagger}$ | $15.5 \pm 0.4^{\dagger}$ |
| $[Ru(CO)_3Cl_2]_2$ + ODQ | $7.1 \pm 1.1^{\dagger}$ | $23.6 \pm 3.8*^{\dagger}$ | $55.5 \pm 6.9*^{\dagger}$ |
| $[Ru(CO)_2(DMSO)_2Cl_2]$ | 1.6 | 16 | 35 |
| $[Ru(CO)_3Cl_2(cytosine)]$ | 3.2 | 10.3 | 12.6 |
| $[Ru(CO)_3(glycinato)Cl]$ | 36 | 66.6 | 68.3 |
| $[Fe(SPh)_2(2,2'-bipyridine)(CO)_2]$ | 50.8 | 60.5 | 75 |
| $[Fe(SPh)_2(H_2NCH_2CH_2NH_2)(CO)_2]$ | 11 | 24.6 | 29.3 |

* $P < 0.01$, compared to vehicle; $^{\dagger}P < 0.01$ compared to $[Ru(CO)_3Cl_2]_2$.

Because CO is thought to modulate signal
transduction mechanisms via increased production of
cGMP, we investigated the effect of a selective
inhibitor of guanylate cyclase (ODQ, 10 µM) on vessel
contractility. As expected, ODQ considerably reduced the
vasodilatation observed after the first two additions of
$[Ru(CO)_3Cl_2]_2$; however, it is of interest that the third
addition of $[Ru(CO)_3Cl_2]_2$ still elicited a substantial

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vasodilatory action despite the presence of ODQ. Thus,
the guanylate cyclase-cGMP pathway appears to be
involved in the relaxation caused by this metal carbonyl
complex.

5

## E.   Expression of heme oxygenase in rat tissues

As a background to the experiments below, we
conducted the following procedure to demonstrate the
effect of stimulating CO production endogenously by
10   treating animals with hemin.

For immunohistochemistry analysis, sections of heart
muscles (5 μm thickness) were treated with 0.3% $H_2O_2$ in
methanol to block endogenous peroxidase activity.
Immunohistochemical staining was performed using rabbit
15   polyclonal antibody against HO-1 (1:1000 dilution) as
previously described (23). The presence of HO-1 was
indicated by the development of a brown color. For
Northern blot analysis, cardiac tissue was ground in a
mortar under liquid nitrogen and suspended in guanidinium
20   thiocyanate lysis buffer. Total RNA was then extracted
using a modification of the method described by
Chomczynski and Sacchi (49).  RNA was run on a 1.3%
denaturing agarose gel containing 2.2 M formaldehyde and
transferred onto a nylon membrane overnight. The
25   membrane was hybridized using [α-$^{32}$P]dCTP-labelled cDNA
probes to rat HO-1 and GAPDH genes and bands analyzed
using a densitometer as previously described (23,50).

Hearts were removed from Lewis rats 24 h after
treatment with vehicle (control) or hemin (75 μmol/kg,
30   i.p.) and immunostaining for HO-1 was assessed.  For
Northern blot analysis, rats were treated with hemin (75
μmol/kg, i.p.) and hearts removed at different time

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points to assess HO-1 mRNA levels (+ve, positive control).

Figure 7 confirms that HO-1 protein (7a) and mRNA (7b) are highly expressed in hearts 24 h after hemin treatment; interestingly, the immunostaining for HO-1 protein was primarily confined to the vessels of cardiac muscle (Figure 7a, right panel).

## F.   Attenuation of vasoconstriction by metal carbonyls in perfused heart

Additional experiments were conducted to determine the biological activity of metal carbonyls on vascular function *in vivo* and compare it with HO-1-derived CO, by monitoring their effects on changes in coronary perfusion pressure (CPP) of isolated rat hearts.

Langendorff heart preparations were performed using male Lewis rats (300-350 g) as previously described by our group (35). Hearts were excised, the aorta cannulated and retrograde perfusion was established at a constant flow of 15 ml/min using Krebs-Henseleit buffer (in mM: 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.66 $MgSO_4$, 24.9 $NaHCO_3$, 1.18 $KH_2PO_4$, 5.55 glucose, 2.00 sodium pyruvate, 0.5 EGTA) bubbled with 95% $O_2$ and 5% $CO_2$ at 37°C (pH 7.4). Coronary perfusion pressure (CPP), a parameter indicative of coronary vessel contractility, was continuously measured by a  pressure transducer connected to the aortic cannula and data analyzed with an Acknowledge software (BIOPAC System Inc.).

Hearts removed either from control rats (vehicle-treated) or from animals that were pre-treated with the heme oxygenase-1 inducer hemin (75 µmol/kg, i.p.) the day before, were initially equilibrated for 20 min on the Langendorff apparatus and then perfused with L-NAME

(25 µM final concentration) to elicit vasoconstriction. The extent of CPP increase by L-NAME was also monitored over time in hemin-treated animals that received a heme oxygenase inhibitor (SnPPIX, 40 µmol/kg) 1 h prior to

5      heart excision and in control hearts that were perfused with buffer supplemented with $[Mn_2(CO)_{10}]$ (13 µM final concentration). Since $[Mn_2(CO)_{10}]$ releases CO only by photodissociation, Krebs buffer containing $[Mn_2(CO)_{10}]$ was exposed to a cold light source immediately before

10     entering the aortic cannula.

        Vasoconstriction was elicited by perfusion with L-NAME and the extent of CPP increase measured over time. As shown in Figure 6, L-NAME caused a time-dependent increase in CPP, which reached a maximum (3-fold) after

15     30 min. Notably, perfusion of hearts with light-stimulated $[Mn_2(CO)_{10}]$ (13 µM) significantly delayed vasoconstriction and maintained CPP at much lower levels at the end of perfusion. When the buffer containing $[Mn_2(CO)_{10}]$ was not exposed to light, thus omitting the

20     CO-induced release process, the extent of constriction mediated by L-NAME was unaffected (data not shown); in addition, perfusion with manganese chloride (negative control) had no effect on myocardial CPP (data not shown).

25         The effect observed with $[Mn_2(CO)_{10}]$ could be similarly reproduced by induction of HO-1 in heart tissue by pretreatment with hemin. It has previously been reported that treatment of rats with hemin results in increased production of cardiac bilirubin, which is

30     equimolar to endogenously generated CO (35). The rise in CPP mediated by L-NAME in hemin-treated hearts was markedly attenuated ($P<0.05$), to an extent similar to that produced by $[Mn_2(CO)_{10}]$ (Figure 6); predictably,

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the effect of hemin was completely reversed by tin
protoporphyrin IX (SnPPIX), a heme oxygenase inhibitor.
Thus, the vasoactive properties of the HO-1/CO pathway
can be simulated by $[Mn_2(CO)_{10}]$.

5       Results are means ± s.e.m. of 6 independent
experiments. * $P<0.05$ vs. vehicle-treated group
(control).


## G.    Animal studies

10      Since it has previously been reported that HO-1-
derived CO also prevents acute hypertension *in vivo*
(40), experiments were performed to examine the
effectiveness of metal carbonyls in regulating mean
arterial pressure in animals.

15      Lewis rats (280-350 g) were anaesthetised by
intramuscular injection of 1 ml/kg Hypnorm (fentanyl
0.315 mg/ml and fluanisone 10 mg/ml) followed 5 min
later by an intraperitoneal injection of 5 mg/kg
diazepam. Specially designed femoral artery and venous

20      catheters were then surgically implanted as previously
described (40). The arterial cannula was connected to a
Grass pressure transducer and blood pressure monitored
continuously using a polygraph recorder. Experiments
were conducted on anaesthetized animals and recordings

25      were made within 30 min of the surgical procedure.
Control rats (vehicle-treated) and animals that were
pre-treated with hemin (75 μmol/kg, i.p) 24 h prior to
blood pressure monitoring were then administered with an
intravenous injection of 30 μmol/Kg L-NAME to elicit an

30      increase in mean arterial pressure. The extent of blood
pressure increase by L-NAME was also monitored over time
in hemin-treated animals that received SnPPIX (40
μmol/kg, i.p.) and in control rats previously injected

with $[Ru(CO)_3Cl_2]_2$ (60 μmol/kg, i.v.). In these two
groups, SnPPIX or $[Ru(CO)_3Cl_2]_2$ were administered to
animals 1 h prior to L-NAME injection. Results are
shown in Figure 8.

5      Intravenous infusion of L-NAME in rats produced a
rapid and significant increase in blood pressure
($P<0.05$); this effect was markedly suppressed by pre-
treatment of animals with a single infusion of
$[Ru(CO)_3Cl_2]_2$ prior to L-NAME administration. Moreover,
10     and in analogy with the data on coronary
vasoconstriction in isolated hearts, treatment of
animals with hemin resulted in a significant suppression
of the L-NAME-mediated hypertensive responses, which
once again was totally reversed by blockade of the heme
15     oxygenase pathway with SnPPIX. Results are the means ±
s.e.m. of 5 independent experiments. *$P<0.05$ vs.
vehicle-treated group (control). Collectively, these *in
vivo* findings attest a consistent and reproducible
biological activity of metal carbonyls through their
20     ability to carry and deliver CO.


## H.    Further studies on CO release

The myoglobin assay procedure of section A above
was carried out on many other metal-carbonyl complexes,
25     to determine the amount of CO release and information on
the kinetics of CO release. The compounds and the
results are tabulated in Figs. 9a to 9f. The compounds
include $[Ru(CO)_3Cl_2]_2$ also tested in section A and
complexes related to it. The applicants' internal
30     reference numbers are used for convenience.

To obtain the data in Figs. 9a to 9f the carbonyl
compounds (CO-RMs) were solubilized in water or DMSO as
indicated and added immediately to a solution of

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myoglobin (66 μM) in phosphate buffer (pH = 7.4). Two different concentrations were tested for each CO-RM (20 and 40 μM) and the conversion of myoglobin to carbon monoxide myoglobin (MbCO) was measured

5   spectrophotometrically at different time points (0, 10, 20 and 30 min). MW = molecular weight. PPT indicates that a precipitate formed. N.D. = "not detectable".

The CO release data of section A above and Figs. 9a to 9f shows that selection of the ligands modulates CO

10   release, both as to amount released and rate of release, permitting selection of release properties, which is important for targeting a specific biological effect.


## I.   Effect of CO-RM-3 (Ru(CO)₃Cl(glycinato)) on systemic

15  ## blood pressure and heart rate in anaesthetised rats

Adult male Sprague-Dawley rats (280 - 350 g, 8 - 10 weeks of age) were bred in-house at the Northwick Park Institute for Medical Research (Harrow, UK). Rats were housed in groups of 3 in cages under a 12 h cycle of

20   day/night, with free access to drinking water and fed *ad libitum*. All surgical procedures were performed in compliance with U.K. Home Office regulations. Rats were anaesthetised in a polycarbonate chamber in a stream of Enflurane™ (Abbot, UK) in oxygen before being

25   transferred onto a mask and continuously supplied with Enflurane™ throughout the experiment with an anaesthetic machine (Airmed, UK). During the surgical procedure the rats were kept at a constant body temperature of 37°C using a heat pad positioned underneath the operating

30   surface. Specially designed femoral artery and venous catheters were then surgically implanted as previously described (see ref. 40). The catheter in the artery was

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connected via a luer connector and a three-way tap to a
pressure transducer (Gould model P23ID, Statham, USA) for
continuous mean arterial pressure (MAP) and heart rate
(HR) monitoring. A purpose-built tail-cuff pressure

5      transducer (ADInstruments, UK) was also placed on the
tail of the rat and pressure transducer and tail cuff
were connected to a polygraph recorder (Grass Model 7D,
Astra-med, UK) pre-calibrated in millimetres of mercury
(mmHg). An analogue output provided data for a computer-

10     based data acquisition system (PowerLab™, ADInstruments,
UK). The computer-based system was set to record mean
arterial pressure (MAP), in mmHg, and heart rate (HR),
in beats/min (bpm), for the duration of the experiment.
A period of 20 minutes was allowed after surgery during

15     which time anaesthetic supply was adjusted so that each
animal had a stable resting MAP of around 80 mmHg ($n=16$,
mean = 81.5 mmHg). Once a stable pressure had been
reached, each catheter was flushed with saline
containing heparin and no further changes were made to

20     the anaesthetic supply. $Ru(CO)_3Cl(glycinato)$ (CO-RM-3)
was prepared in stock solutions of 20, 60 and 120
$\mu moles.ml^{-1}$ by solubilizing the compound in saline. $Cis$-
$RuCl_2(DMSO)_4$, which does not contain any carbonyl groups,
was used as a 'negative control'. CO-RM-3 (or the

25     negative control) was then infused into the animal via
the femoral vein catheter as a bolus so that the final
concentration infused was 10, 30 or 60 $\mu moles.kg^{-1}$ body
weight. Throughout the experiment MAP and HR were
continuously recorded and monitored. Although

30     concentrations of 10, 30 and 60 $\mu moles.kg^{-1}$ were infused
into each animal, the resulting concentrations in the
animal were cumulative. Therefore, the final

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concentration attained in the animal was 10, 40 and 100
$\mu$moles.kg$^{-1}$, respectively.

The results are presented in Table 2, where the
data shown represents samples taken at baseline (just
5   before infusion of the compound) and directly after
administration of 10, 30 and 60 $\mu$moles.kg$^{-1}$ of the
compound. All data are mean $\pm$ SEM. $n$ = 3 independent
experiments. *P<0.05 vs. baseline.

*Cis*-RuCl$_2$(DMSO)$_4$ (control) had no significant
10   effects on either HR or MAP at any of the concentrations
used (10, 30 or 60 $\mu$moles.kg$^{-1}$). Even after the final
(60 $\mu$moles.kg$^{-1}$) infusion of *Cis*-RuCl$_2$(DMSO)$_4$, the MAP
(81 $\pm$ 4 mmHg) and HR (256 $\pm$ 9 bpm) were well preserved
compared to baseline measurements (80 $\pm$ 2 mmHg and
15   257 $\pm$ 7 bpm, respectively). There was a marginal
increase (5.5 $\pm$ 1 mmHg) in MAP during the administration
of each bolus of the compound. However, this effect is
believed to be associated with a volume increase since
it also occurred when saline was infused during the
20   inserting procedure. In contrast, administration of
Ru(CO)$_3$Cl(glycinato) resulted in a concentration-
dependent transient decrease in MAP followed by a return
to baseline over a period of 10 min; with 10, 30 and 60
$\mu$moles.kg$^{-1}$ bolus infusions resulting in 6 $\pm$ 2, 8 $\pm$ 3
25   and 14 $\pm$ 0.3 (*P*<0.05) mmHg decreases, respectively. As
before, HR remained unchanged (253 $\pm$ 23 bpm) compared to
baseline (270 $\pm$ 20 bpm). These data demonstrate that CO
liberated from CO-RM-3 can modulate blood pressure and
can be used therapeutically to control acute and chronic
30   hypertensive responses *in vivo*. These data parallel the
evidence that endogenous CO generated from activated

heme oxygenase-1 is a potent vasodilator and suppresses acute hypertension *in vivo* (see ref. 23 and 40).

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Table 2

| Compound | Baseline | | 10 µmol.kg$^{-1}$ | | 30 µmol.kg$^{-1}$ | | 60 µmol.kg$^{-1}$ | |
|---|---|---|---|---|---|---|---|---|
| | HR (bpm) | MAP (mmHg) | HR (bpm) | MAP (mmHg) | HR (bpm) | MAP (mmHg) | HR (bpm) | MAP (mmHg) |
| Cis-RuCl$_2$(DMSO)$_4$ (negative control) | 257 ± 7 | 80 ± 2 | 255 ± 6 | 82 ± 4 | 256 ± 8 | 79 ± 5 | 256 ± 9 | 81 ± 4 |
| Ru(CO)$_3$Cl(glycinato) (CO-RM-3) | 270 ± 20 | 79 ± 2 | 276 ± 20 | 73 ± 2 | 261 ± 22 | 72 ± 7 | 253 ± 23 | 65 ± 4* |

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## J.   Effect of Ru(CO)₃Cl(glycinato) on cardiac transplant rejection in mice

Hearts from male BALB/c mouse (25-30 g) were used as donor organs for transplantation into male CBA mice

5    (25-30 g). Mice were housed in groups of 3 in cages under a 12 h cycle of day/night, with free access to drinking water and fed *ad libitum*. All surgical procedures were performed in compliance with U.K. Home Office regulations. Animals were anaesthetized by an

10    intraperitoneal injection of ketamine/xylazine during all procedures. The surgical technique involved the transplantation of the cardiac allograft into the recipient's neck as previously described (51). Graft survival was assessed daily by palpation, and rejection

15    was diagnosed by cessation of ventricular contractions.

Ru(CO)₃Cl(glycinato) was dissolved in 0.1 ml saline and administered intraperitoneally. All doses are 40 mg/kg of Ru(CO)₃Cl(glycinato). The donors received two doses of Ru(CO)₃Cl(glycinato) respectively at 1 day and

20    15 min prior to cardiac harvest. The recipients received doses of Ru(CO)₃Cl(glycinato) at 1 day before surgery, 30 min prior to cardiac reperfusion and 1 h after transplantation (Day 0). Thereafter, graft recipients received a daily dose of Ru(CO)₃Cl(glycinato)

25    from day 1 to day 8 (inclusive) post-transplant. In the control group, recipients received an equivalent dose of saline (vehicle) 1 day before and each day (days 1 to 8) after cardiac transplantation. Carprofen (0.01 mg) was given subcutaneously for pain relief immediately after

30    transplantation to all animals. The results of this study are shown in Figure 10. n = 5 for each group. *p<0.002 vs. control. BALB/c hearts trasplanted into

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CBA mice following treatment with saline (control group) underwent rejection very rapidly. 100% of hearts stopped beating within 9 days of transplantation. In contrast, the survival time of hearts transplanted into mice receiving Ru(CO)$_3$Cl(glycinato) was significantly prolonged (p<0.002) with 100% of hearts still beating 18 days after transplantation. At 25 days after heart transplantation, 60% of mice treated with Ru(CO)$_3$Cl(glycinato) still did not show any sign of rejection (p<0.002) and at 30 days 40% of transplanted hearts were still viable. These data demonstrate that Ru(CO)$_3$Cl(glycinato) is very effective in prolonging the survival of murine cardiac grafts and attenuating organ rejection. The result is in parallel with recently published reports showing that mice treated with CO gas (by inhalation) are significantly less susceptible to graft rejection in a model of mouse-to-rat cardiac transplant (51).

Based on the findings above on CO release and vasorelaxation, the data in this section indicates that CO liberated from the carbonyl complex mediates the anti-rejection process.

### K. Effect of Ru(CO)$_3$Cl(glycinato) on nitric oxide production in macrophages following endotoxin challenge

The signaling molecule nitrogen monoxide (NO), which is generated in mammals by a family of constitutive (nNOS and eNOS) and inducible (iNOS) NO synthase enzymes, plays an essential regulatory role in a variety of physiological and pathophysiological processes that take place within the cardiovascular, nervous and immune systems (52). Overproduction of NO

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has been established as a potent cytotoxic weapon in
host defense against infection, inflammation and cancer.
Considerable amounts of NO can originate from activated
iNOS when appropriately induced by cytokines, endotoxins
5    or lipopolysaccharide (LPS), oxygen free radicals or
other stressful stimuli (53). In particular,
macrophages are a specific target of pro-inflammatory
stimuli as they highly express iNOS and can generate
excessive amounts of NO to modulate important
10   cytostatic/bactericidal actions. From unpublished data,
it has been postulated that induction of the heme
oxygenase-1 (HO-1)/bilirubin/CO pathway represents a
counter-regulatory system against the deleterious
effects elicited by overproduction of NO. Specifically,
15   both CO and bilirubin may interfere with NO generation
by acting as inhibitors of NOS activity and scavenger of
NO, respectively. CO gas has been shown to inhibit NOS
activity in various tissues (54), and it has been
suggested that bilirubin can directly interact with NO
20   and NO-related species (55).

The present study was undertaken to assess the
effect of Ru(CO)$_3$Cl(glycinato) (CO-RM-3) on the
production of NO from endotoxin-stimulated macrophages.
Mouse RAW 264.7 macrophages were cultured in 24 wells
25   using DMEM medium. Confluent cells were incubated for
24 h with *E. Coli* lipopolysaccharide (LPS, 3 µg/ml) in
the presence or absence of increasing concentrations of
CO-RM-3 (10, 50 and 100 µM). Control cells were exposed
to culture medium alone. Nitrite in the culture medium
30   was measured as an index of NO production using the
Griess reagent method (56). Cell viability was also
assessed in macrophages 24 h after treatment with the

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various agents as described in reference 47.  Treatment
of macrophages with LPS caused a significant increase in
nitrite levels (p<0.05) after 24 h incubation (see Fig.
11, where bars represent the mean ± S.E.M. of 6
5    independent experiments.  *p<0.05 vs. control; [†]p<0.05
vs. LPS.  The presence of CO-RM-3 significantly
attenuated nitrite generation in a concentration
dependent manner.  As shown in Fig. 12 (where bars
represent the mean ± S.E.M. of 6 independent
10    experiments), these treatments did not affect cell
viability as no toxic effect was observed at the end of
the incubation period.

     These data indicate in the ability of CO released
from CO-RM-3 to prevent the inflammatory response in
15    macrophages by inhibiting the production of iNOS-derived
NO.  Furthermore, and in line with the beneficial
effects shown by CO-RM-3 on blood pressure and cardiac
graft rejection, these results suggest a potential
therapeutic application of water-soluble CO carriers in
20    the modulation of vascular- and inflammatory-related
pathological states.


## L.   Syntheses

     Synthetic methods for obtaining compounds of Figs.
25    9a to 9f tested for CO release will now be described.
Purity of the product has not been investigated in
detail.  Stereoisomers are expected to be present.


**Preparation of $Ru(CO)_3Cl(NH_2CH_2\{CH_2SH\}CO_2)$ [$M_R$ 340.5]**

30    L-Cysteine complex.  Reference number: CO-RM-26
     $[Ru(CO)_3Cl_2]_2$ (0.129g, 0.25 mmol) and L-cysteine
(0.039g, 0.50 mmol) were placed under nitrogen in a

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round bottomed flask. Methanol (75 cm$^3$) and sodium
ethoxide (0.034g, 0.50 mmol) were added and the reaction
allowed to stir for 18 hours. The solvent was then
removed under pressure and the yellow residue

5   redissolved in THF, filtered and excess 40-60 light
petroleum added. The yellow solution was evaporated
down to given an orange solid (0.120g, 70%).

**Preparation of Ru(CO)$_3$Cl(NH$_2$CH$_2$CO2) [M$_R$ 294.5]**

10  Glycine complex. Reference number: CO-RM-3
        [Ru(CO)$_3$Cl$_2$]$_2$ (0.129g, 0.25 mmol) and glycine
(0.039g, 0.50 mmol) were placed under nitrogen in a
round bottomed flask. Methanol (75 cm$^3$) and sodium
ethoxide (0.034g, 0.50 mmol) were added and the reaction

15  allowed to stir for 18 hours. The solvent was then
removed under pressure and the yellow residue
redissolved in THF, filtered and excess 40-60 light
petroleum added. The yellow solution was evaporated
down to give a pale yellow solid (0.142g, 96%).

20

**Preparation of Ru(CO)$_3$Cl(NH$_2$CH{CHMeCH$_2$CH$_3$}CO$_2$) [M$_R$ 350.5]**
DL-Isoleucine complex. Reference number: CO-RM-38
        [Ru(CO)$_3$Cl$_2$]$_2$ (0.129g, 0.25 mmol) and DL-isoleucine
(0.066g, 0.50 mmol) were placed under nitrogen in a

25  round bottomed flask. Methanol (75 cm$^3$) and sodium
ethoxide (0.034g, 0.50 mmol) were added and the reaction
allowed to stir for 18 hours. The solvent was then
removed under pressure and the yellow residue
redissolved in THF, filtered and excess 40-60 light

30  petroleum added. The yellow solution was evaporated
down to give a yellow solid (0.086g, 49%).

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**Preparation of Ru(CO)$_3$Cl(NH$_2$CH{CH$_2$OH}CO$_2$) [M$_R$ 324.5]**

L-Serine complex.  Reference number:  CO-RM-39.

[Ru(CO)$_3$Cl$_2$]$_2$ (0.129g, 0.25 mmol) and L-serine
(0.053g, 0.50 mmol) were placed under nitrogen in a
5      round bottomed flask.  Methanol (75 cm$^3$) and sodium
ethoxide (0.034g, 0.50 mmol) were added and the reaction
allowed to stir for 18 hours.  The solvent was then
removed under pressure and the yellow residue
redissolved in THF, filtered and excess 40-60 light
10     petroleum added.  The yellow solution was evaporated
down to give a pale yellow solid (0.095g, 59%)

**Preparation of Ru(CO)$_3$Cl(NH$_2$CH{CH$_3$}CO$_2$ [M$_R$ 308.5]**

L-Alanine complex.  Reference number:  CO-RM-40.

15        [Ru(CO)$_3$Cl$_2$]$_2$ (0.129g, 0.25 mmol) and L-alanine
(0.045g, 0.50 mmol) were placed under nitrogen in a
round bottomed flask.  Methanol (75 cm$^3$) and sodium
ethoxide (0.034g, 0.50 mmol) were added and the reaction
allowed to stir for 18 hours.  The solvent was then
20     removed under pressure and the yellow residue
redissolved in THF, and filtered.  The solution was
evaporated down to give an orange solid (0.145g, 94%).

**Preparation of Ru(CO)$_3$Cl(NH$_2$CH{CH$_2$CH$_2$CONH$_2$}CO$_2$) [M$_R$ 365.5]**

25     L-Glutamine complex.  Reference number:  CO-RM-42.

[Ru(CO)$_3$Cl$_2$]$_2$ (0.129g, 0.25 mmol) and L-glutamine
(0.073g, 0.50 mmol) were placed under nitrogen in a
round bottomed flask.  Methanol (75 cm$^3$) and sodium
ethoxide (0.034g, 0.50 mmol) were added and the reaction
30     allowed to stir for 18 hours.  The solvent was then
removed under pressure and the yellow residue
redissolved in THF and filtered.  The solution was

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evaporated down to give a yellow oil which solidified under high vacuum to give a pale yellow solid (0.170g, 93%).

5    **Preparation of RU(CO)₃Cl(NH₂CH{CH₂CH₂NHC(=NH)NH₂}CO₂)**
        **[M$_R$ 393.5]**

L-Arginine complex. Reference number: CO-RM-43.

[Ru(CO)₃Cl₂]₂ (0.129g, 0.25 mmol) and L-arginine (0.087g, 0.50 mmol) were placed under nitrogen in a
10   round bottomed flask. Methanol (75 cm³) and sodium ethoxide (0.034g, 0.50 mmol) were added and the reaction allowed to stir for 18 hours. The solvent was then removed under pressure and the yellow residue redissolved in THF/MeOH (4:1) and filtered. The
15   solution was evaporated down to given an orange solid (0.185g, 94%).

**Preparation of Ru(CO)₃Cl(NH₂CH{CH₂CH₂CH₂CH₂NH₂}CO₂)**
        **[M$_R$ 365.5]**

20   L-Lysine complex. Reference number: CO-RM-46.

[Ru(CO)₃Cl₂]₂ (0.129g, 0.25 mmol) and L-lysine (0.073g, 0.50 mmol) were placed under nitrogen in a round bottomed flask. Methanol (75 cm³) and sodium ethoxide (0.034g, 0.50 mmol) were added and the reaction
25   allowed to stir for 18 hours. The solvent was then removed under pressure and the yellow residue redissolved in THF/MeOH (3:1) and filtered. The solution was evaporated down to give a yellow oil which solidified under high vacuum to given an orange solid
30   (0.163g, 89%).

**Preparation of Ru(CO)₃Cl(NH₂CH{CH(CH₃)₂}CO₂[M$_R$ 336.5]**

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L-Valine complex.   Reference number: CO-RM-67.

$[Ru(CO)_3Cl_2]_2$ (0.129g, 0.25 mmol) and L-valine
(0.059g, 0.50 mmol) were placed under nitrogen in a
round bottomed flask.  Methanol (75 cm$^3$) and sodium

5   ethoxide (0.034g, 0.50 mmol) were added and the reaction
allowed to stir for 18 hours.  The solvent was then
removed under pressure and the yellow residue
redissolved in THF and filtered.  Excess 40-60 light
petroleum was added and the solution evaporated down to

10  given a white solid (0.114g, 68%).


**Preparation of Ru(CO)$_3$Cl(NH$_2$CH{CH(OH)CH$_3$}CO$_2$) [M$_R$ 338.5]**
L-Threonine complex.  Reference number: CO-RM-74.

$[Ru(CO)_3Cl_2]_2$ (0.129g, 0.25 mmol) and L-threonine

15  (0.060g, 0.50 mmol) were placed under nitrogen in a
round bottomed flask.  Methanol (75 cm$^3$) and sodium
ethoxide (0.034g, 0.50 mmol) were added and the reaction
allowed to stir for 18 hours.  The solvent was then
removed under pressure and the yellow residue

20  redissolved in THF and filtered.  Excess 40-60 light
petroleum was added and the solution evaporated down to
give a white solid (0.149g, 88%).


**Preparation of [Fe(η-C$_5$H$_5$)(CO)$_3$]Cl [M$_R$ 240.5]**

25  Reference number: CO-RM-70.

A sodium amalgam was prepared by, under nitrogen in
a Schlenk tube, dissolving sodium metal (2.04g) in
mercury (18 cm$^3$).  This was allowed to cool to room
temperature and tetrahydrofuran (40 cm$^3$) added.  Then

30  [FeCp(CO)$_2$]$_2$ (7.08g, 20.3 mmol) in tetrahydrofuran (60
cm$^3$) added and the flask shaken vigorously for 45
minutes.

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Then into a large 3-necked flask purged with
nitrogen, THF (300 cm$^3$) and ethyl chloroformate (40 mmol,
4.34g, 3.84 cm$^3$) were placed and cooled to 0°C.  The red-
yellow solution of cleaved dimer was then transferred
5       into the round bottomed flask and allowed to stir for
one hour at low temperature before being concentrated in
volume.  The red-brown residue was extracted with
benzene (5 x 20 cm$^3$), the extracts filtered, and HCl gas
blown through the solution for 15 minutes.  An immediate
10      precipitation was observed, the solution was reduced in
volume and the orange precipitate collected, washed with
diethyl ether (20 cm$^3$) and dried.  (4.84g, 50%).


**Preparation of [Fe(η-C$_5$H$_5$)(CO)$_3$]PF$_6$[M$_R$ 350]**

15      Reference number: CO-RM-71.

[Fe(η-C$_5$H$_5$)(CO)$_3$]Cl (3.00g, 12.5 mmol) was dissolved
in water (50 cm$^3$) and sodium hexafluorophosphate (2.00g,
1eq) in water (50 cm$^3$) added.  An orange precipitate was
immediately formed, the reaction stirred for 15 minutes
20      and the orange precipitate collected under suction
(3.04g, 70%).


**Preparation of Ru(CO)$_3$Cl$_2$(guanosine) [M$_R$ 540]**

Reference number: CO-RM-17.

25      [Ru(CO)$_3$Cl$_2$]$_2$ (0.129g, 0.25 mmol) and guanosine
(0.142g, 0.50 mmol) were placed under nitrogen in a
round bottomed flask.  Methanol (75 cm$^3$) was added and
the reaction allowed to stir for 18 hours.  The solution
was then filtered and reduced in volume to approximately
30      10 cm$^3$.  Excess diethyl ether was added and the white
precipitate formed allowed to settle out in the freezer
overnight.  The solvent was pipetted off to leave a

50

white solid which was dried under high vacuum (0.130g,
48%).


**Preparation of [Ru(CO)₃Cl(guanosine)₂]/Cl[M_R 824]**

5    Reference number: CO-RM-18.

         [Ru(CO)₃Cl₂]₂ (0.129g, 0.25 mmol) and guanosine
     (0.284g, 1.00 mmol) were placed under nitrogen in a
     round bottomed flask. Methanol (75 cm³) was added and
     the reaction allowed to stir for 18 hours. The solution

10   was then filtered and reduced in volume to approximately
     10 cm³. Excess diethyl ether was added and the white
     precipitate formed allowed to settle out in the freezer
     overnight. The solvent was pipetted off to leave a
     white solid which was dried under high vacuum (0.220g,

15   53%).


**Preparation of Ru(CO)₃Cl₂(triacetyl-guanosine) [M_R 666]**
     Reference number: CO-RM-29.

         [Ru(CO)₃Cl₂]₂ (0.129g, 0.25 mmol) and 2, 3, 5

20   -triacetylguanosine (0.205g, 0.50 mmol) were placed
     under nitrogen in a round bottomed flask. Methanol (75
     cm³) was added and the reaction allowed to stir for 18
     hours. The solution was then filtered and reduced in
     volume to approximately 10 cm³. Excess diethyl ether was

25   added and the white precipitate formed allowed to settle
     out in the freezer overnight. The solvent was pipetted
     off to leave a white solid which was dried under high
     vacuum (0.212g, 63%).


30   **Preparation of Ru(CO)₃Cl₂(guanine) [M_R 408]**
     Reference number: CO-RM-22.

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$[Ru(CO)_3Cl_2]_2$ (0.129g, 0.25 mmol) and guanine
(0.076g, 0.50 mmol) were placed under nitrogen in a
round bottomed flask. Tetrahydrofuran (75 cm$^3$) was added
and the reaction allowed to stir for 18 hours. The
5    solution was then reduced in volume to approximately 10
cm$^3$. Excess 40-60 light petroleum was added and the
precipitate formed allowed to settle out in the freezer
overnight. The solvent was pipetted off to leave a pale
yellow solid which was dried under high vacuum (0.082g,
10   39%).


**Preparation of [Ru(CO)$_3$Cl(guanine)$_2$]Cl [M$_R$ 558]**
Reference number: CO-RM-23.
        $[Ru(CO)_3Cl_2]_2$ (0.129g, 0.25 mmol) and guanine
15   (0.152g, 1.00 mmol) were placed under nitrogen in a
round bottomed flask. Tetrahydrofuran (75 cm$^3$) was added
and the reaction allowed to stir for 18 hours. The
solution was then reduced in volume to approximately 10
cm$^3$. Excess 40-60 light petroleum was added and the
20   precipitate formed allowed to settle out in the freezer
overnight. The solvent was pipetted off to leave a
cream solid which was dried under high vacuum (0.170g,
61%).


25   **Preparation of *fac*-RuCl$_2$(CO)$_3$(THF) [M$_R$ 328]**
Reference number: CO-RM-11.
        $[Ru(CO)_3Cl_2]_2$ (0.380g, 0.74 mmol) and tetrahydrofuran
(5 cm$^3$) were placed in a conical flask and the yellow
solution stirred for 15 minutes. Then the solvent was
30   removed under reduced pressure leaving a yellow oil
which upon standing solidified. Addition of
diethylether (20 cm$^3$) accompanied by sonication afforded

a white precipitate and yellow solution. The solid was
collected and dried under vacuum (0.134g, 28%).


**Preparation of $[RuCl_2(CO)_2]_n[M_R$ unknown]**

5    Reference number: CO-RM-10.

    $RuCl_3xH_2O$ (5.00g), concentrated hydrochloric acid
(25 cm$^3$) and formic acid (25 cm$^3$) were placed in a 3-
necked round bottomed flask and the mixture refluxed for
18 hours. The clear yellow solution was then reduced in
10   volume to leave a yellow/orange precipitate, which was
transferred into a Soxhlet thimble and extracted
overnight with methanol. This solution was then reduced
in volume to give an orange oil which solidified under
high vacuum to afford an orange precipitate (5.30g).

15

**Preparation of $Ru(CO)_3(O\{CH_2CO_2\}_2)$ [$M_R$ 317]**

Diglycolic acid complex. Reference number: CO-RM-99

    $[Ru(CO)_3Cl_2]_2$ (0.129g, 0.25 mmol) and diglycolic
acid (0.067g), 0.50 mmol) were placed under nitrogen in
20   a round bottomed flask. Methanol (75 cm$^3$) and sodium
ethoxide (0.068g, 1.00 mmol) were aded and the reaction
allowed to stir for 18 hours. The solvent was then
removed under pressure and the yellow residue
redissolved in THF, filtered and excess 40-60  light
25   petroleum added. The yellow solution was evaporated
down to give a white solid (0.142g, 85%).


**Preparation of $Ru(CO)_3(NH\{CH_2CO_2\}_2)$ [$M_R$ 317]**

Iminodiacetic acid complex. Reference number: CO-RM-97
30   $[Ru(CO)_3Cl_2]_2$ (0.129g, 0.25 mmol) and iminodiacetic
acid (0.066g, 0.50 mmol) were  placed under nitrogen ina
round bottomed flask. Methanol (75 cm$^3$) and sodium

ethoxide (0.068g, 1.00 mmol) were added and the reaction allowed to stir for 18 hours. The solvent was then removed under pressure and the yellow residue redissolved in THF/MeOH (4:1), filtered and excess 40-60

5   light petroleum added. The yellow solution was evaporated down to give an off-white solid (0.140g, 89%).


Syntheses suitable for CO-RM-1a, CO-RM-1b and the

10  negative controls for these compounds are in reference 57. Synthesis of CO-RM-16 is found in reference 58.

While the invention has been described in conjunction with the exemplary embodiments described above, many equivalent modifications and variations will

15  be apparent to those skilled in the art when given this disclosure. Accordingly, the exemplary embodiments of the invention set forth above are considered to be illustrative and not limiting. Various changes to the described embodiments may be made without departing from

20  the spirit and scope of the invention.

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## CLAIMS:

1. A pharmaceutical composition, for delivery of carbon monoxide to a physiological target, comprising a metal carbonyl compound or pharmaceutically acceptable salt thereof and at least one pharmaceutically acceptable carrier, wherein the metal carbonyl makes available CO suitable for physiological effect by at least one of the following means:

1) CO derived by dissociation of the metal carbonyl is present in the composition in dissolved form;

2) on contact with a solvent the metal carbonyl releases CO;

3) on contact with a tissue, organ or cell the metal carbonyl releases CO;

4) on irradiation the metal carbonyl releases CO.

2. A pharmaceutical composition according to claim 1, wherein the metal carbonyl compound is a complex of at least one of Fe, Mn, Ru, Rh, Ni, Mo or Co with at least one carbonyl ligand.

3. A pharmaceutical composition according to claim 1 or claim 2, wherein the metal is bound to at least one group other than CO.

4. A pharmaceutical composition according to claim 3, wherein the group other than CO is a modulatory group, which modulates the solubility of the compound and/or the release of CO from the compound.

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5.    A pharmaceutical composition according to any one
of claims 1 to 4 adapted for administration by an oral,
intravenous, subcutaneous, nasal, inhalatory,
intramuscular, intraperitoneal or suppository route.

5

6.    A pharmaceutical composition for delivery of CO,
comprising as active ingredient a compound of the
formula $M(CO)_x A_y$ where x is at least one, y is at least
one, M is a metal, the or each A is an atom or group
10    bonded to M by an ionic, covalent or coordination bond,
and in the case where y>1 each A may be the same or
different, or a pharmaceutically acceptable salt of such
a compound.

15    7.    A pharmaceutical composition according to claim 6
wherein M is a transition metal.

8.    A pharmaceutical composition according to claim 6
or claim 7, wherein A is selected from halogens, groups
20    having N, P, O or S atoms providing lone electron pairs
for coordination bonding to M, and conjugated carbon
groups.

9.    A pharmaceutical composition according to any one
25    of claims 6 to 8 adapted for delivery by an oral,
intravenous, subcutaneous, nasal, inhalatory,
intramuscular, intraperitoneal or suppository route.

10.   A method of introducing CO to a mammal as a
30    physiologically effective agent comprising the step of
administering a pharmaceutical composition according to
any one of claims 1 to 9.

11.   A method according to claim 10, for the stimulation
of guanylate cyclase activity.

12.   A method according to claim 10 or claim 11, for
stimulating neurotransmission or vasodilation, or for
the treatment of any of hypertension, radiation damage,
endotoxic shock, inflammation, an inflammatory-related
disease, hyperoxia-induced injury, apoptosis, cancer,
transplant rejection, arteriosclerosis, post-ischemic
organ damage, myocardial infarction, angina,
haemorrhagic shock, sepsis, penile erectile dysfunction
and adult respiratory distress syndrome.

13.   Use of a metal carbonyl compound in the manufacture
of a medicament for administration by an oral,
intravenous, subcutaneous, nasal, inhalatory,
intramuscular, intraperitoneal, or suppository route,
for the stimulation of neurotransmission or vasodilation
by CO as a physiologically effective agent, or for the
treatment of any of hypertension, radiation damage,
endotoxic shock, inflammation, inflammatory-related
diseases, hyperoxia-induced injury, apoptosis, cancer,
transplant rejection, arteriosclerosis, post-ischemic
organ damage, myocardial infarction, angina,
haemorrhagic shock, sepsis, penile erectile dysfunction
and adult respiratory distress syndrome.

14.   Use according to claim 13 wherein the metal
carbonyl compound is a complex of at least one of Fe,
Mn, Ru, Rh, Ni, Mo or Co with at least one carbonyl
ligand.

15.   Use of a metal carbonyl compound as set out in any one of claims 6 to 8 in the manufacture of a medicament for the stimulation of neurotransmission or

5    vasodilation, or for the treatment of any of hypertension, radiation damage, endotoxic shock, inflammation, an inflammatory-related disease, hyperoxia-induced injury, apoptosis, cancer, transplant rejection, arteriosclerosis, post-ischemic organ damage,

10   myocardial infarction, angina, haemorrhagic shock, sepsis, penile erectile dysfunction and adult respiratory distress syndrome.

16.   A method of treating a viable mammalian organ

15   extracorporeally comprising contacting the organ with a pharmaceutical composition according to any one of claims 6 to 8.

17.   A metal carbonyl compound of the formula

20          $M(CO)_x A_y B_z$ where

M is Fe, Co or Ru,

x is at least one,

y is at least one,

z is zero or at least one,

25          each A is a ligand other than CO and is monodentate or polydentate with respect to M and is selected from the amino acids

alanine

arginine

30          asparagine

aspartic acid

cysteine

glutamic acid

glutamine

glycine

histidine

5          isoleucine

leucine

lysine

methionine

phenylalanine

10         proline

serine

threonine

tryptophan

tyrosine

15         valine

$O(CH_2COO)_2$ and

$NH(CH_2COO)_2$, and

B is optional and is a ligand other than CO,

excluding $Fe(CO)_x A_y$ where A is cysteine or an ester

20  of cysteine and $Ru(CO)_xAy$ where A is proline.


18.  A pharmaceutical composition, for delivery of

carbon monoxide to a physiological target, comprising a

compound which is formic acid, a formate, a formate

25  ester or amide, an oxalate or an oxalate ester or amide,

or a pharmaceutically acceptable salt thereof, and at

least one pharmaceutically acceptable carrier, wherein

the compound makes available CO suitable for

physiological effect.

30

19.    A method of introducing CO to a mammal comprising
the step of administering a pharmaceutical composition
according to claim 18.


5    20.    A method according to claim 19 for stimulating
guanylate cyclase activity, neurotransmission or
vasodilation, or for the treatment of any of
hypertension, radiation damage, endotoxic shock,
inflammation, an inflammatory-related disease,
10    hyperoxia-induced injury, apoptosis, cancer, transplant
rejection, arteriosclerosis, post-ischemic organ damage,
myocardial infarction, angina, haemorrhagic shock,
sepsis, penile erectile dysfunction and adult
respiratory distress syndrome.
15

Dimanganese decacarbonyl
[$Mn_2(CO)_{10}$]

Iron pentacarbonyl
[$Fe(CO)_5$]

Fig. 1

Fig. 2(a)

Fig. 2(b)

Fig. 2(c)

Fig. 2(d)

Fig. 2(e)



Fig. 2(f)

Tricarbonyldichloro ruthenium(II) dimer
[Ru (CO)₃(Cl)₂]₂

1                         2                         3
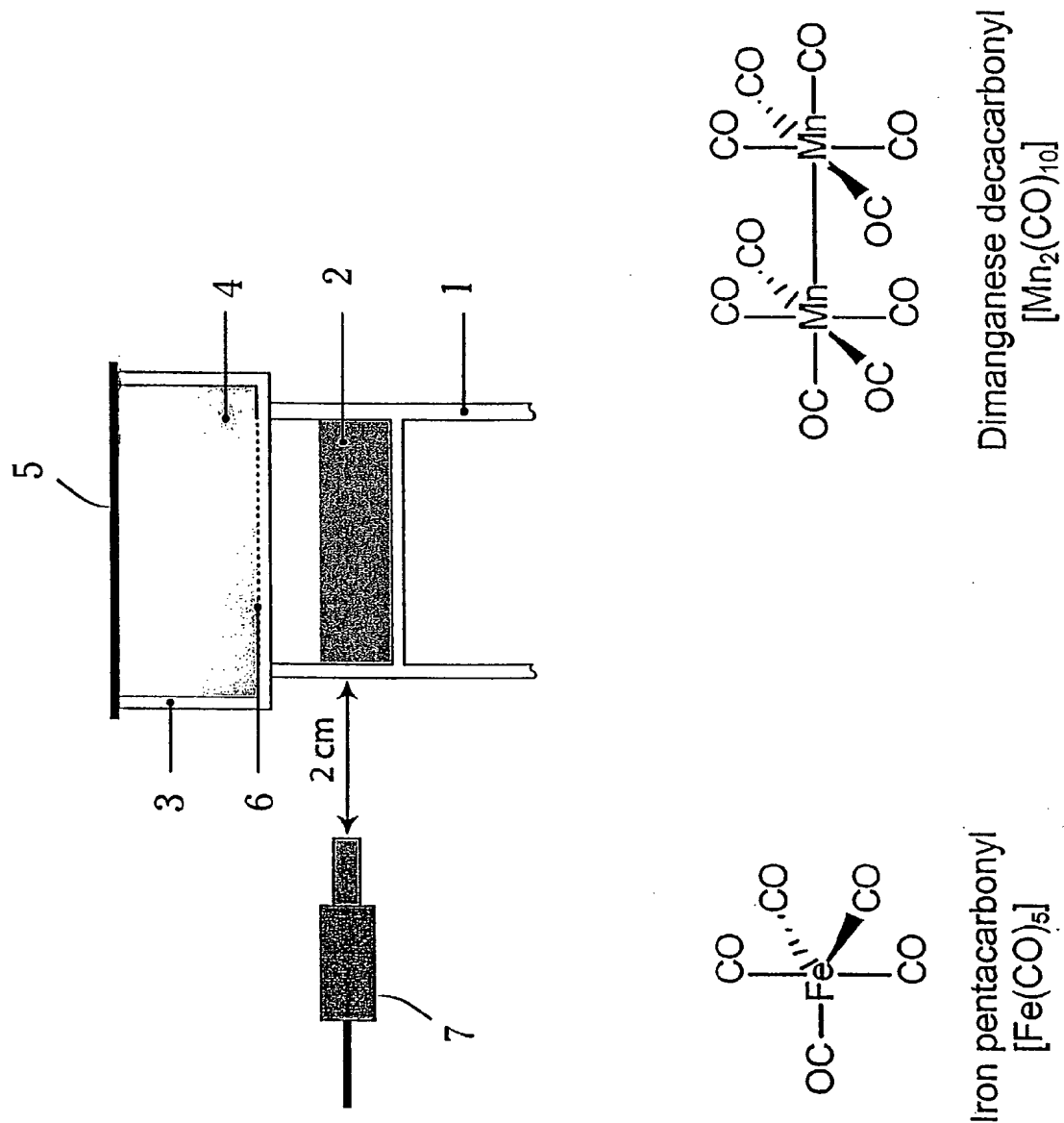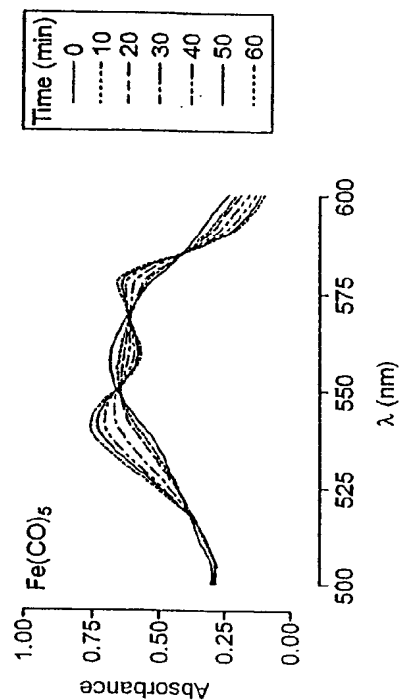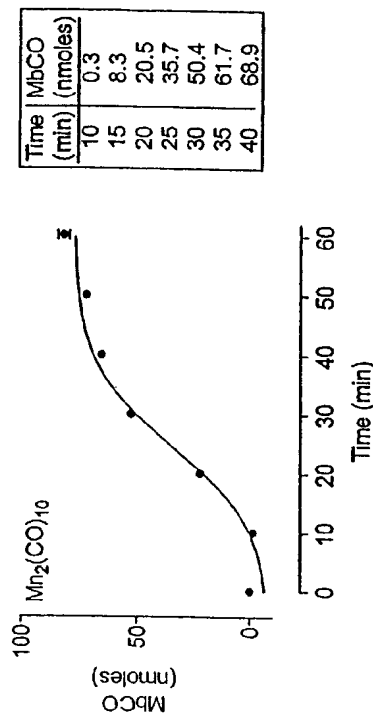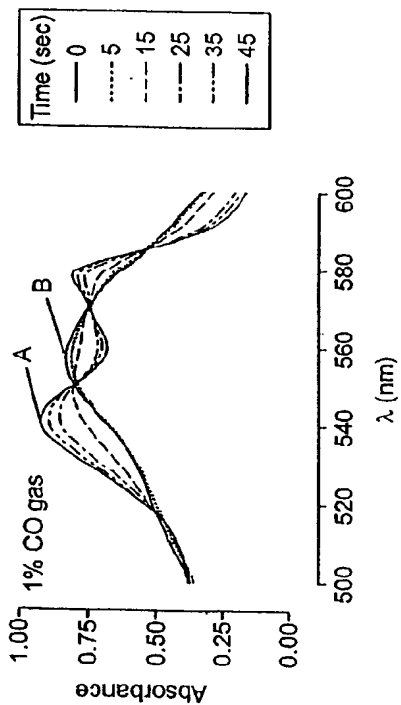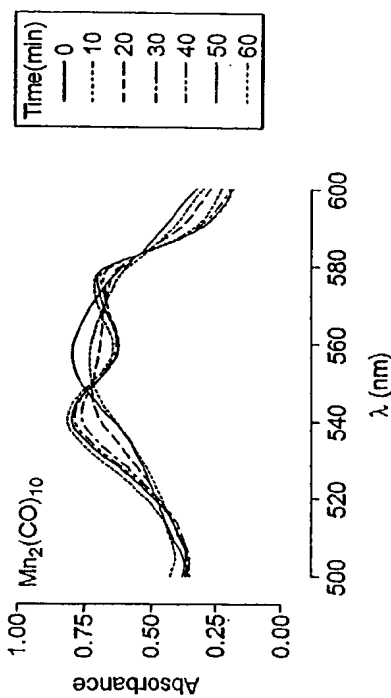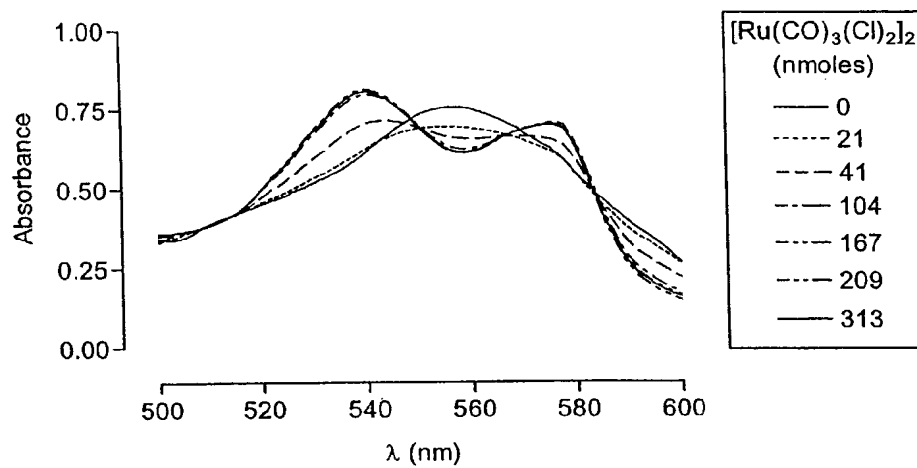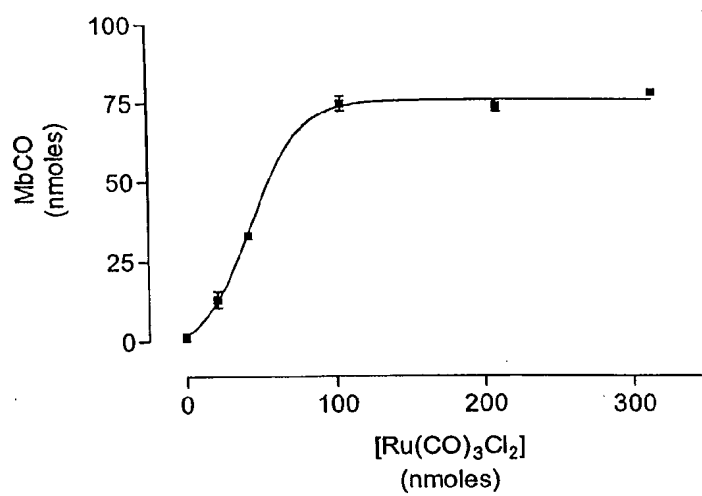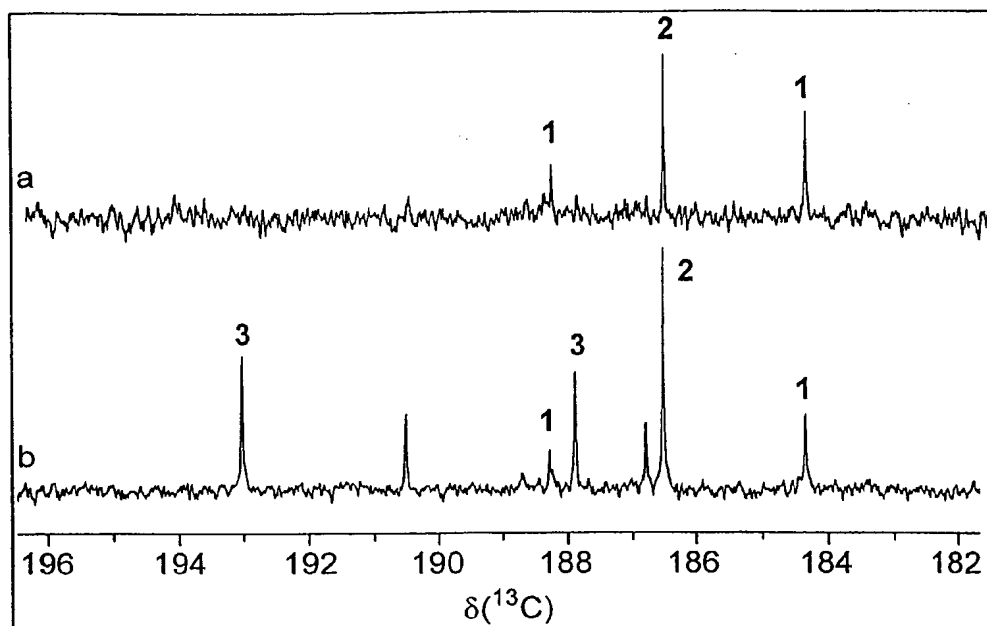
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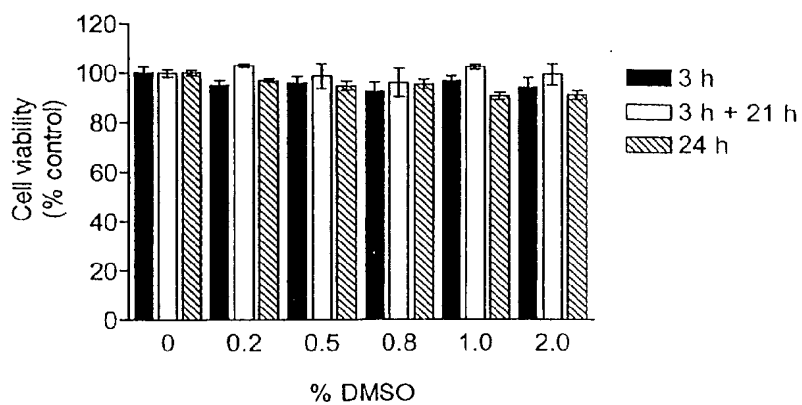
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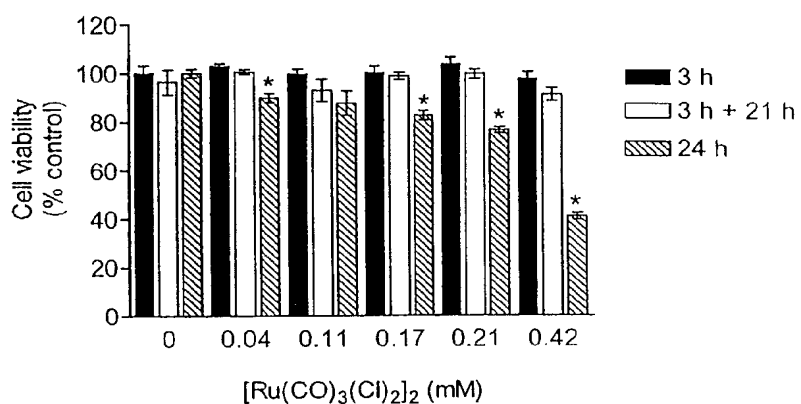


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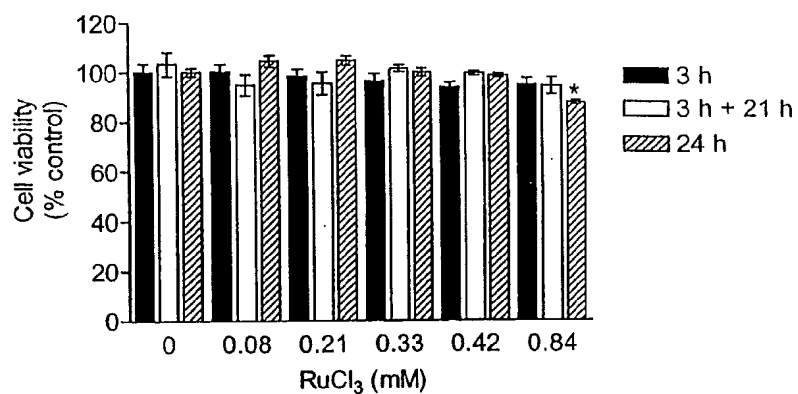


Fig. 4(c)
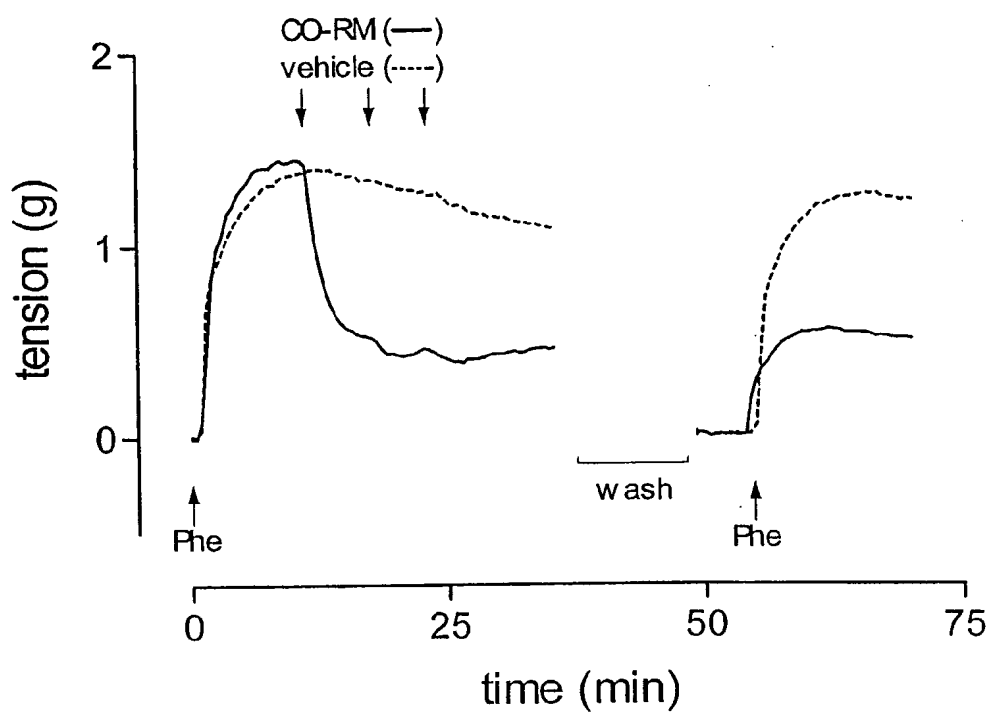
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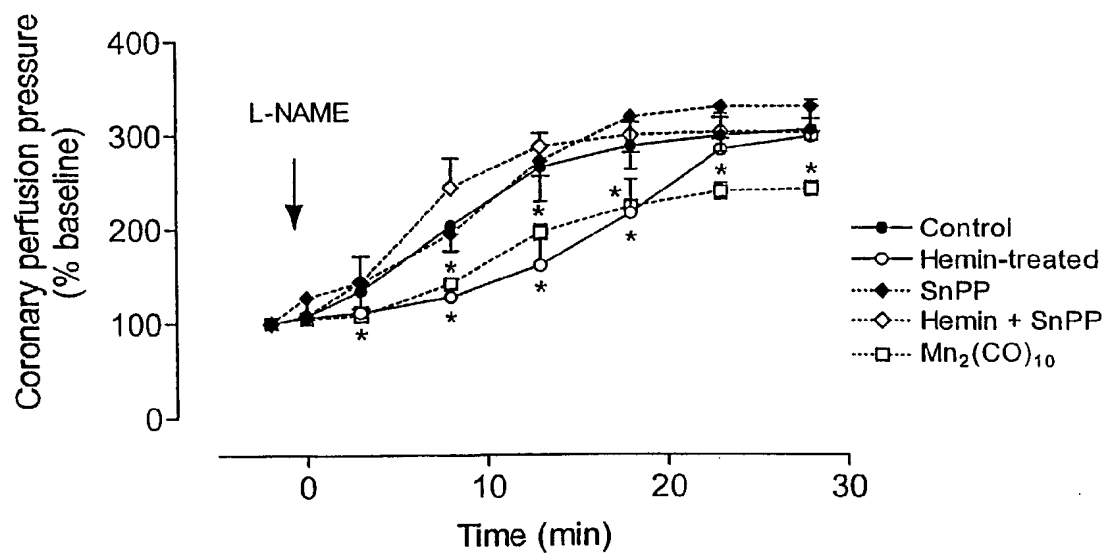


Fig. 6
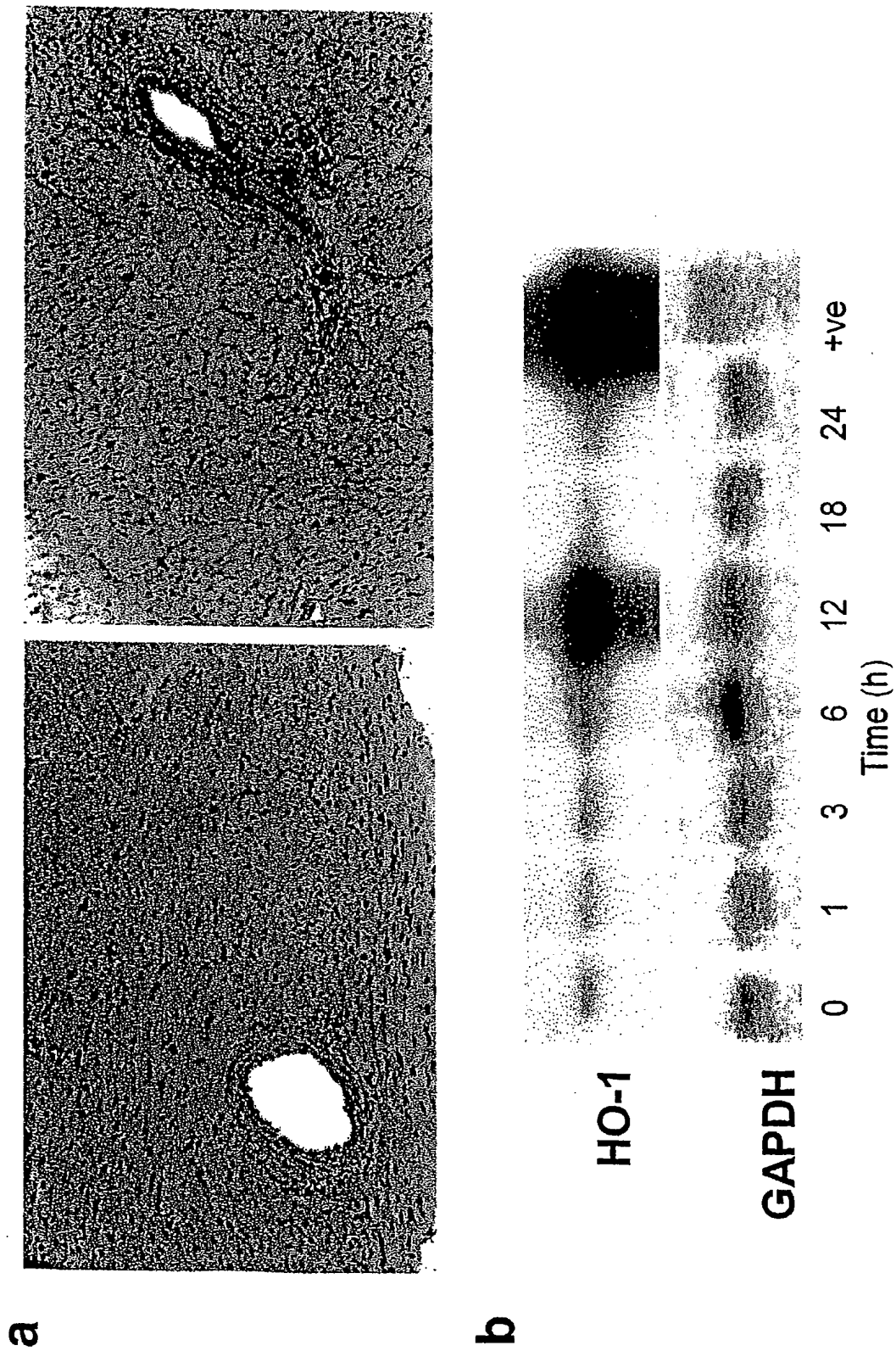
Fig. 7
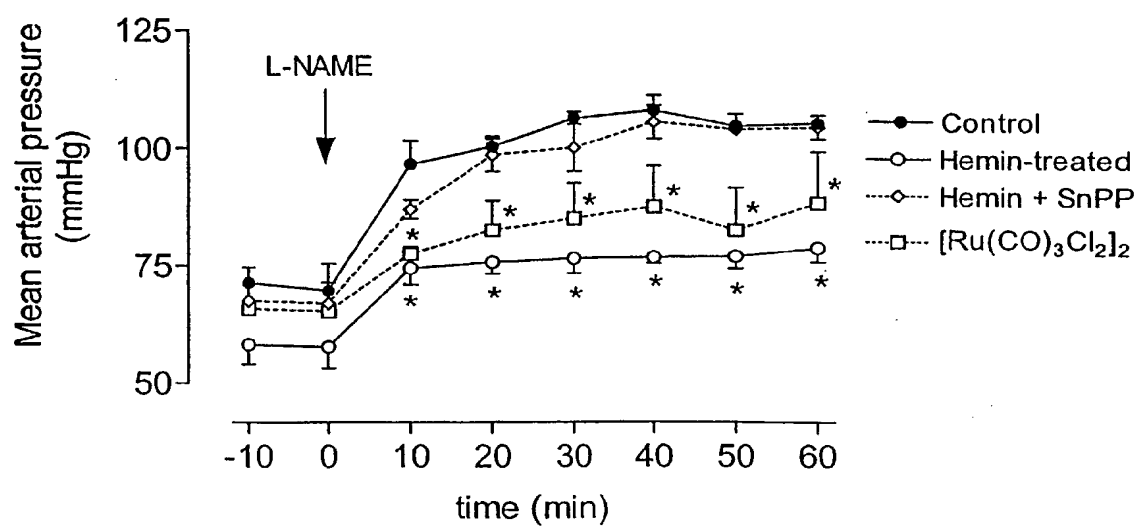
Fig. 8

| Compound | Structure | MW | CO Release (20 µmoles) | | | | CO Release (40 µmoles) | | | | NOTES |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 10 | 20 | 30 | 0 | 10 | 20 | 30 | |
| CO-RM-1 | (Ru dimer structure) | 512 | 12.0 ±3.0 | 16.3 ±4.0 | 18.1 ±4.3 | 18.5 ±4.8 | 28.5 ±0.4 | 32.0 ±0.2 | 34.5 ±0.5 | 35.6 ±0.4 | Soluble in DMSO |
| CO-RM-1a | (Ru complex structure) | 384 | 7.2 ±0.6 | 8.6 ±0.3 | 8.0 ±0.4 | 7.5 ±0.4 | 16.9 ±0.6 | 18.4 ±0.3 | 17.3 ±0.3 | 16.7 ±0.2 | Soluble in DMSO |
| Negative control | (Ru complex structure) | 484 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | Soluble in H$_2$O |
| CO-RM-1b | (Ru complex structure) | 334 | 6.4 ±1.2 | 7.3 ±0.6 | 8.2 ±0.1 | 8.7 ±0.3 | 11.7 ±0.8 | 13.7 ±0.9 | 14.0 ±1.1 | 14.4 ±0.6 | Soluble in DMSO |
| CO-RM-10 | $\left[ Ru(CO)_2Cl_2 \right]_n$ | (228) | 2.6 ±0.6 | 9.8 ±0.3 | 12.7 ±0.1 | 13.8 ±0.9 | 8.6 ±0.7 | 21.0 ±1.1 | 24.4 ±1.0 | 26.3 ±1.2 | Soluble in DMSO |

Fig. 9(a)

Fig. 9(b)
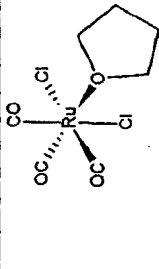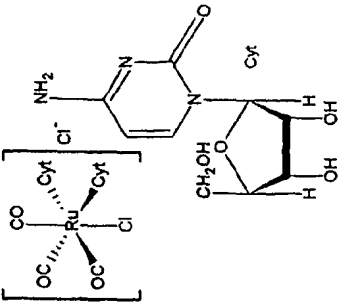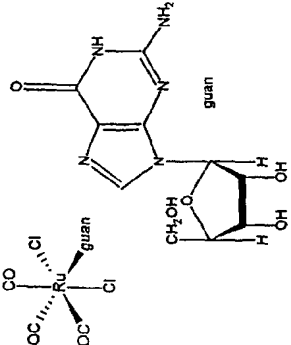
| Compound | Structure | MW | | | | | | | | | Solubility |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **CO-RM-11** Ligand: THF | | 328 | 5.6 ±0.6 | 5.9 ±0.6 | 6.2 ±1.1 | 6.2 ±1.2 | 10.9 ±0.2 | 12.3 ±0.4 | 13.3 ±0.4 | 13.7 ±0.2 | Soluble in DMSO |
| **CO-RM-16** Ligand: Cytidine | | 742 | N.D. | 1.4 ±0.4 | 2.1 ±0.1 | 2.8 ±0.4 | 0.8 ±0.4 | 5.5 ±0.4 | 8.4 ±0.8 | 9.8 ±0.9 | Soluble in $H_2O$ |
| **CO-RM-17** Ligand: Guanosine | | 539 | 5.9 ±0.1 | 8.2 ±0.4 | 8.5 ±0.3 | 8.6 ±0.4 | 11.5 ±0.4 | 15.0 ±0.4 | 15.6 ±0.4 | 16.2 ±0.3 | Soluble in $H_2O$ |

| | | | | | | | | | Solubility |
|---|---|---|---|---|---|---|---|---|---|
| **CO-RM-18** Ligand: Guanosine | 822 | 10.1 ±0.9 | 14.3 ±0.4 | 14.1 ±0.5 | 13.5 ±0.4 | 25.4 ±1.0 | 29.5 ±1.5 | 29.5 ±1.4 | 28.7 ±1.3 | Soluble in $H_2O$ |
| **CO-RM-22** Ligand: Guanine | 407 | 0.1 ±0.1 | 0.8 ±0.3 | 1.0 ±0.3 | 2.3 ±0.1 | 0.7 ±0.1 | 1.9 ±0.1 | 2.3 ±0.1 | 2.4 ±0.1 | Soluble in $H_2O$ **PPT** |
| **CO-RM-23** Ligand: Guanine | 558 | 1.2 ±0.1 | 1.3 ±0.2 | 1.3 ±0.1 | 1.0 ±0.2 | 2.7 ±0.3 | 2.7 ±0.3 | 2.7 ±0.4 | 2.3 ±0.2 | Soluble in $H_2O$ **PPT** |
| **CO-RM-26** Ligand: Cysteine | 340.5 | 0.6 ±0.1 | 1.9 ±0.1 | 2.3 ±0.2 | 2.4 ±0.2 | 1.9 ±0.2 | 3.7 ±0.1 | 5.1 ±0.1 | 5.2 ±0.1 | Soluble in $H_2O$ |
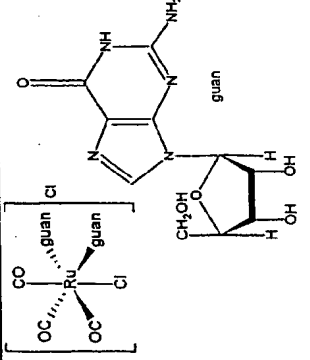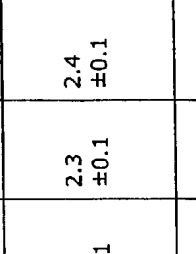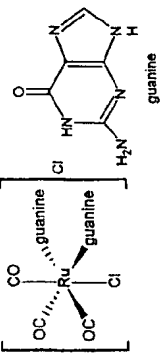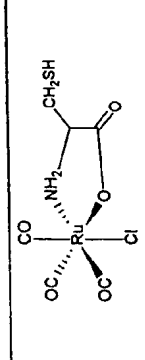
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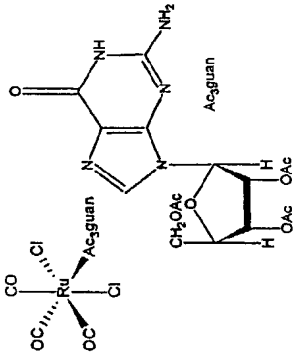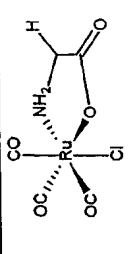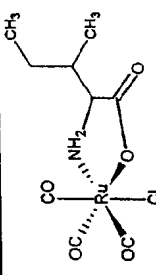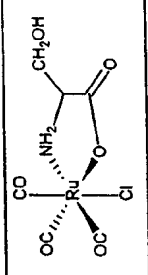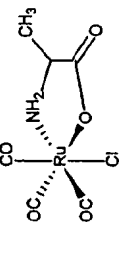
| | MW | | | | | | | | | Soluble in H₂O |
|---|---|---|---|---|---|---|---|---|---|---|
| **CO-RM-29** Ligand: Triacetyle-guanosine | 665 | 1.4 ±0.7 | 4.5 ±0.1 | 5.0 ±0.1 | 3.2 ±0.1 | 8.3 ±0.6 | 11.7 ±0.3 | 12.4 ±0.1 | 10.6 ±0.4 | Soluble in $H_2O$ |
| **CO-RM-3** Ligand: Glycine | 294.5 | 14.2 ±0.6 | 17.8 ±0.7 | 14.3 ±0.7 | 12.9 ±0.7 | 25.2 ±1.5 | 24.4 ±1.0 | 23.8 ±0.6 | 23.2 ±0.3 | Soluble in $H_2O$ |
| **CO-RM-38** Ligand: Isoleucine | 350.5 | 3.2 ±0.2 | 4.4 ±0.1 | 4.0 ±0.2 | 3.0 ±1.7 | 7.6 ±1.3 | 8.3 ±1.2 | 7.5 ±1.1 | 7.3 ±1.1 | Soluble in $H_2O$ |
| **CO-RM-39** Ligand: Serine | 324.5 | 11.0 ±.03 | 12.8 ±.09 | 11.4 ±1.1 | 10.8 ±.07 | 24.2 ±1.5 | 24.6 ±1.4 | 22.0 ±1.0 | 21.9 ±1.2 | Soluble in $H_2O$ |
| **CO-RM-40** Ligand: Alanine | 308.5 | 9.1 ±1.1 | 11.9 ±0.4 | 11.1 ±.03 | 11.0 ±0.2 | 20.2 ±.06 | 21.3 ±.09 | 19.9 ±.09 | 19.6 ±.09 | Soluble in $H_2O$ |

Fig. 9(d)
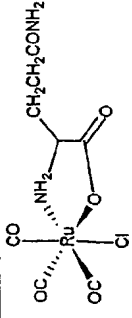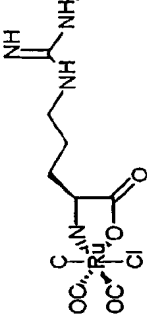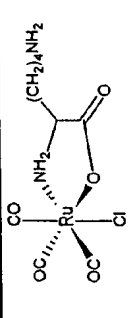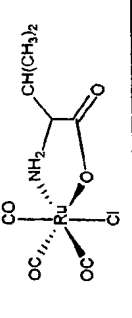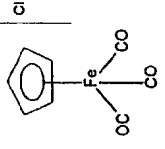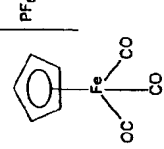
| Compound / Ligand | Structure | MW | | | | | | | | | Solubility |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CO-RM-42 Ligand: Glutamine | | 365.5 | 8.9 ±0.4 | 11.1 ±0.4 | 12.1 ±1.4 | 10.1 ±0.3 | 21.4 ±2.1 | 21.8 ±2.2 | 20.6 ±2.0 | 20.0 ±1.8 | Soluble in $H_2O$ |
| CO-RM-43 Ligand: Arginine | | 393.5 | 9.4 ±1.4 | 11.9 ±0.5 | 12.3 ±0.7 | 11.0 ±0.3 | 18.3 ±.03 | 20.0 ±0.6 | 19.0 ±1.2 | 17.8 ±1.3 | Soluble in $H_2O$ |
| CO-RM-46 Ligand: Lysine | | 365.5 | 6.0 ±0.4 | 7.5 ±0.8 | 7.2 ±1.2 | 6.4 ±0.8 | 12.6 ±0.9 | 13.4 ±1.2 | 13.2 ±1.1 | 11.9 ±1.0 | Soluble in $H_2O$ |
| CO-RM-67 Ligand: L-valine | | 336.5 | 11.1 ±2.9 | 18.2 ±1.7 | 17.6 ±1.6 | 17.0 ±1.6 | 29.3 ±1.5 | 34.6 ±2.2 | 33.7 ±2.2 | 32.8 ±2.2 | Soluble in $H_2O$ |
| CO-RM-70 | | 240 | 0.5 ±0.2 | 0.9 ±0.1 | 2.2 ±0.2 | 2.7 ±0.3 | 0.9 ±0.1 | 2.0 ±0.2 | 4.9 ±0.2 | 6.3 ±0.3 | Soluble in DMSO **PPT** |
| CO-RM-71 | | 350 | 1.5 ±0.2 | 2.3 ±0.3 | 3.1 ±0.4 | 3.7 ±0.4 | 3.4 ±0.1 | 5.4 ±0.3 | 6.9 ±0.3 | 7.6 ±0.4 | Soluble in DMSO **PPT** |

Fig. 9(e)
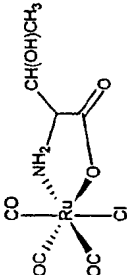
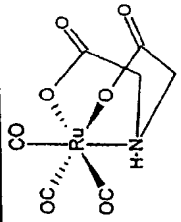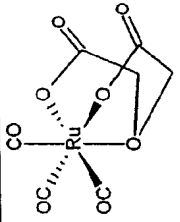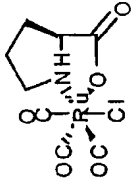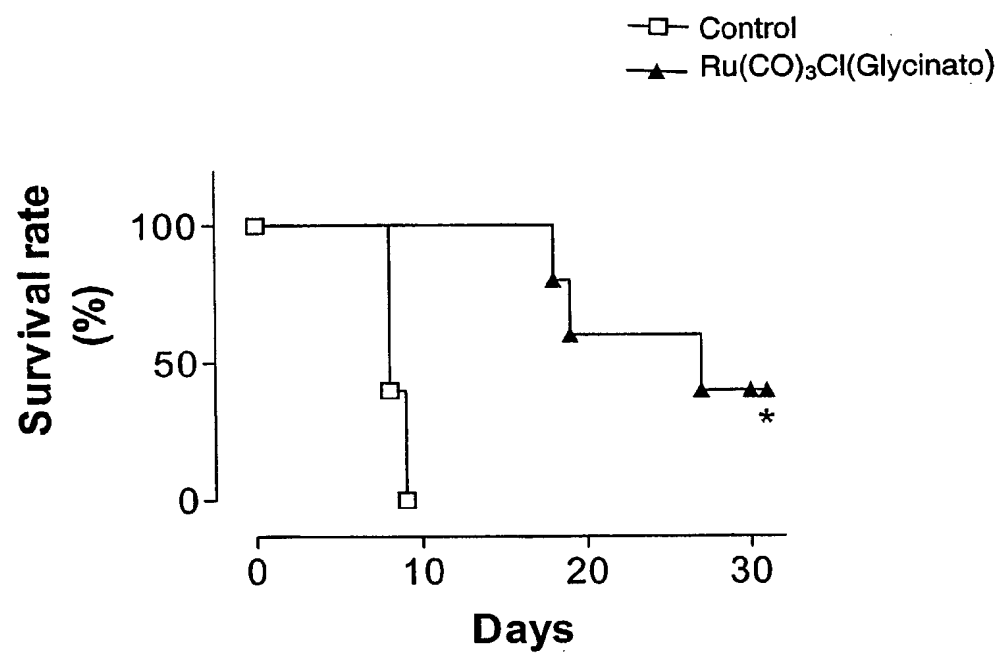| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CO-RM-74 Ligand: L-Threonine |  | 338.5 | 15.7 ±1.2 | 17.5 ±2.0 | 16.5 ±2.3 | 14.8 ±2.2 | 33.3 ±0.2 | 33.4 ±0.1 | 32.7 ±0.2 | 31.4 ±0.1 | Soluble in H₂O |
| CO-RM-97 |  | 316 | 2.8 ± 0.6 | 7.0 ± 0.7 | 7.2 ± 0.9 | 6.6 ± 0.9 | 7.1 ± 0.5 | 14.3 ± 0.7 | 14.7 ± 0.8 | 13.6 ± 0.7 | Soluble in H₂O |
| CO-RM-99 |  | 317 | 4.6 ± 0.6 | 8.1 ± 0.2 | 7.3 ± 0.3 | 5.5 ± 0.3 | 11.5 ±0.2 | 16.6 ± 0.2 | 16.0 ± 0.9 | 14.0 ± 0.2 | Soluble in H₂O |
| CO-RM-H Ligand: L-proline |  | 335 | 1.4 ± 0.3 | 4.7 ± 0.6 | 6.2 ± 0.8 | 6.3 ± 0.7 | 4.2 ±0.4 | 9.9 ± 0.2 | 12.5 ± 0.1 | 13.0 ±0.1 | Soluble in H₂O |

Fig. 9(f)

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Fig. 10

Fig. 11

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Fig. 12